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Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Berzelius and Pharmacy*

By GEORGE URDANG†

IT WAS a century ago, on the seventh of August, 1848, that scientists all over the civilized world mourned the death of one of the greatest among the many excellent chemists who, at the end of the eighteenth and during the first half of the nineteenth centuries, systematized and utilized the new views opened by Antoine Laurent Lavoisier (1743–1794). This man was the Swede, Jöns Jacob Berzelius (1779–1848).

This is not the place to list in detail the numerous contributions to chemistry which have earned immortality for the name of Berzelius. It may suffice to state that it was not only investigations based on thoughts of his own, comprehensive and of highest importance as they were, but his evaluation, classification, and supplementation of the chemical findings of his contemporaries which made the work of Berzelius so enormously influential and consequential.

Some Contributions of Berzelius.—On the basis of the suggestions presented by the Frenchmen J. H. Hassenfratz (1755–1827) and P. A. Adet (1763–1832) the great Swede created the chemical symbols which, with some changes, are still in use. He coined the terms allotropy and catalysis, thus harnessing the phenomena concerned into well-defined limits and chemical tools. He investigated the chemical proportions in a way never surpassed

Jöns Jacob Berzelius (1779–1848)

* Presented before the joint session of the A. PH. A. Section on Historical Pharmacy and the American Institute of the History of Pharmacy at San Francisco on August 14, 1948.

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and proved the validity of the law of proportions not only for inorganic, but also for organic substances. Together with W. Hisinger (1766–1852) he presented the fundamentals of the electrochemical theory more than four years before Humphry Davy (1778–1829) published his epoch-making respective observations.

Pharmaceutical Scientists about 1800.—The chemical period into which Berzelius was born was decidedly influenced by great pharmaceutical scientists. In his own country, Sweden, it had been the apothecary-chemist, Carl Wilhelm Scheele (1742–1786), whose early death had prematurely closed an unusually brilliant chain of discoveries. In France, Louis Nicolas Vauquelin (1763–1829), likewise growing into research from the ranks of pharmacy, was president of the Parisian Academy of Science when Berzelius, in 1819, in a special session of this famous institution presented the French edition of his book on the electrochemical theories and chemical proportions, and Berzelius proudly reports in his autobiographical notes[1] the compliments paid to him by this great apothecary. In Germany, the chemical scene in the late eighteenth and early nineteenth centuries was dominated by the pharmacist, Martin Heinrich Klaproth (1743–1817), whom Berzelius, in his reference to a priority quarrel concerning the discovery of cerium, called "Europe's greatest analytical chemist."[2] There was, furthermore, quite a galaxy of other prominent apothecary-chemists around 1800 everywhere, especially in France and Germany, but also in England and in Russia.

This situation offers an explanation for the fact that, whenever there seemed to be a vague possibility of a practical pharmaceutical activity for the youth Berzelius, it was seized and dwelt upon, and that statements to this effect have been made now and again.

It was in order to contradict the idea of Berzelius having been a pharmacist that Theodor Husemann (1833–1901) in an article written on the occasion of the fiftieth anniversary of the death of the great chemist made the following statements:

"Berzelius, unlike several other famous northern naturalists...was not a pharmacist by profession. He was a physician and, after having completed his studies, he has indeed practiced medicine (as physician at Werner's mineral water establishment at Stockholm) from 1803 to 1806 besides acting as an 'adjunct to the professor of medicine and pharmacy,' a position with an entirely insufficient remuneration."[3]

Pharmaceutical Experience of the Youth Berzelius.—However, although Berzelius never was "a pharmacist by profession," were there not indeed relations of a substantial nature entitling pharmacy to claim some modest part in the development of the great scientist? The fact that the brief notes on Berzelius in J. A. Häfliger's "Biographicon" include the remark "worked in a pharmacy"[4] seems to justify such an assumption. It is furthermore underlined by the following passage in H. G. Söderbaum's book on "Berzelius Development and Growth, 1779–1821":

"Idleness was something which the restless nature of Berzelius could stand the least. He longed for taking up experimental work again and in order to satisfy this longing he decided to look for a position in the pharmacy of some neighboring town. At this time Berzelius was the guest of his aunt Bromander at Bromma near Motala. He remembers a relative and namesake who is an apothecary in Jönköping. He applies for a job, receives, however, a negative reply. He is more successful in Vadstena. There he stays in the pharmacy for the rest of the summer (1799) *and takes part in the entire pharmaceutical work*.[5] This episode, as unessential as it may appear as such, is nevertheless of some interest since it furnishes proof for the fact how well Berzelius already at this early time understood how to take advantage of whatever came into his way and to utilize it in one way or another. The insight into the practical part of the profession of pharmacy which he gained in the plain Vadstena apothecary shop was by no means lost. On the contrary, it offered the first incentive to his publication of thirteen years later entitled 'On the Chemical Preparations in the Pharmacopœias of the Various Countries' which made him *a reformer of Swedish pharmacy*.[6] His later activity in this field has, by the way, by no means been without influence on the organization of the public health system in other countries."[7]

It is in all probability these statements which still in 1948, in the most recent Berzelius biography, written by Wilhelm Prandtl, found their repercussion in a passage telling that during the summer vacation in 1799 the student Berzelius went to the country, "where life was less expensive and occasionally money could be earned by teaching or *as a clerk in a pharmacy*."[8]

Do the real facts conform with the description of this epsiode in Berzelius' life and the conclusions drawn by the enthusiastic biographer H. G. Söderbaum? The view given by the one who ought to know, Berzelius himself, is quite different, and it is not without an ironical connotation that the same man who wrote the flowery story quoted above was bound to bring his hero's contradictory statements to the knowledge of the world. In 1901, only two years after his first book on Berzelius had appeared, H. G. Söderbaum edited for the Royal Swedish Academy of Science the "Autobiographical Notes" of the great chemist presented by the latter to the Academy. In these "Notes" Berzelius describes his vacation activities of the year 1799 as follows:

"I had an uncle, Daniel Berzelius, who was an apothecary in Jonköping, of whom I asked leave to help in the apothecary shop during the summer. In this way I hoped to acquire distinct pharmaceutical experience, but he refused my request. I then accepted the invitation of my aunt, Mrs. Flora Bromander, to spend the summer with her family at

[1] H. G. Söderbaum, J. J. Berzelius, "Autobiographical Notes," translated into English by Olof Larsell, Baltimore 1934, p. 40; Berzelius quotes Vauquelin as follows: "We consider it as only a proper reparation to say to you that, if your and Mr. Hisinger's work on the chemical effects of the electric pile had been known to us when Davy received the great prize from us, we should have divided it between you and him."

[2] *Ibid.*, p. 51.

[3] *Pharm. Ztg.*, **43**, 63(1898).

[4] J. A. Häfliger, Biographicon, in A. Tschirch, "Handbuch der Pharmakognosie," ed. 2, Leipzig, 1931/33, vol. 7, part 2.

[5,6] For the italicized statements Söderbaum refers to M. Pontin, "Biographi," p. 205. Magnus Pontin (1781–1858), since 1835 physician in ordinary to the King of Sweden, was a lifelong friend and, for some time, collaborator of Berzelius.

[7] H. G. Söderbaum, Berzelius' "Werden und Wachsen, 1779–1821," Leipzig, 1899.

[8] Wilhelm Prandtl, "Humphry Davy—J. J. Berzelius," Stuttgart, 1948, p. 122. Prandtl lists Söderbaum's book on Berzelius among his sources.

Bromma near Motala. Bromander was a very industrious man.... He soon found me much too idle which was not to his taste. He sent me, therefore, after a few weeks to Vadstena where I was to live with his mother, a likable but quite elderly lady. I was also to work with apothecary Wessel who, incorrectly, was considered a skillfull chemist. In his pharmacy there was little to do. But there was an Italian who blew glass and made barometers, a good old man named Josua Vaccano. With him I associated myself and learned to blow glass with a burner, as well as to make barometers and thermometers. With this occupation I busied myself at home between the hours of instruction from him. After a month I returned again to Bromma."[9]

This statement in which Berzelius himself stresses the lack of opportunity to do pharmaceutical work in the Vadstena apothecary shop and mentions the information in the art of glass blowing as the main achievement of this vacational month, deprives this epoch in the life of the great chemist of every pharmaceutical significance.

The difference between the real meaning of the episode in Vadstena as to Berzelius' later pharmaceutical knowledge and interests, however, and the interpretation given to it by his biographer offers an excellent example for one of the most frequent sources of mistaken views in so many biographies: the endeavor to construe some causal connection between every event in the life of the hero with all subsequent ones which seem to develop along the same line. In his later life Berzelius took an active and very beneficial interest in pharmacy. In his younger life he had spent some time in a pharmacy. There had to be a causal nexus between these two facts.

It is interesting to note that another and much more important job, done by Berzelius in a pharmacy only one year after his pharmaceutically rather uneventful stay at Vadstena, has not been given much publicity by those eager to establish an early influence of pharmacy on Berzelius. In the summer of 1800 Berzelius, still only a candidate of medicine, held for some months the position of a physician to the poor at Medevi, a then much frequented Swedish spa. He evaporated the water of the various springs and, leaving the examination of the residues for the time after his return to Upsala, he made "gas analyses on the spot in the apothecary's shop."[10] This proves that the pharmacy at Medevi offered the young chemist the adequate opportunity, apparatus, and reagents for his analysis which, by the way, has to be considered as his first scientific accomplishment and served him as basis for his dissertation. There is, however, no evidence for the assumption that this use of the facilities of an apothecary shop for scientific work had any influence on the attitude of Berzelius toward pharmacy.

Besides, there is no necessity for any attempt at finding some more or less farfetched reason for the activities of Berzelius in the interest of pharmacy. It belonged, after all, to his duties as a professor of chemistry and pharmacy to pay attention to the scientific fundamentals of the latter. Another factor was that, as intimated above, at this time chemistry and pharmacy were still to a very great extent undivided.

When the young student Berzelius applied for admittance into the laboratory of his chemistry professor at Upsala in order to start practical chemical work, he was given "the two thick volumes" of the renowned "Textbook on the Art of Pharmacy" ("Lehrbuch der Apothekerkunst") by the apothecary and professor of pharmacy at Königsberg in Prussia, Carl Gottfried Hagen. The accompanying advice was: "read them first."[11] The two first preparations to be assigned to the ambitious student were undoubtedly of a pharmaceutico-chemical nature, namely, *Colcothar Vitrioli*, an impure iron oxide obtained by heating iron sulfate, and potassium carbonate obtained by heating cream of tartar.

It is a peculiar accident that it was just the pharmaceutical examination to be passed at that time by the Swedish candidates for the degree of Doctor of Medicine which became a cause of great irritation to Berzelius, not because of any fault or deficiency on his part, but because of personal differences among the respective professors at the University of Upsala.

Berzelius the Pharmacy Teacher.—In 1803, only one year after he had met the requirements for the medical doctorate—the official bestowal of the degree did not take place until midsummer 1804—Berzelius was appointed "Adjunct in Medicine and Pharmacy" at the School of Surgery at Stockholm.[12] In this capacity he gave lectures on chemistry to which "the pupils at the School of Surgery...as also the students of pharmacy were invited to listen...."[13] In 1806 he introduced at the College of Medicine at Stockholm "exercises in pharmaceutical[14] and chemical operations similar to those in Upsala." In 1807 Berzelius was named as professor of medicine and pharmacy at the School of Surgery, his duties comprising the teaching of chemistry, general science (called at that time "natural history"), pharmacy, and medicine. In 1809, finally, the instruction pertaining to the professorship held by Berzelius "was divided among three new professors,"[15] leaving to Berzelius only the two disciplines in which he was interested primarily, chemistry first and then, in its relations to this science, pharmacy. In 1811 Berzelius was given a seat and a voice in the Swedish College of Medicine and it is, for the topic of this paper, of interest that he received this appointment in his capacity as professor of pharmacy.

In 1812, one year after the firm establishment of Berzelius as the academic representative of Swedish pharmacy, he published the aforementioned observations "On the Chemical Preparations in the Pharmacopœias of the Various Countries." The subsequent activities of the great Swede in the interest and for the sake of pharmacy not only in his homeland but in the world at large have been described by Husemann as follows:

"In his capacity as a professor of pharmacy Berzelius has helped the Swedish pharmacists not

[9] H. G. Söderbaum, J. J. Berzelius, "Autobiographical Notes," *loc. cit.*, p. 28.
[10] *Ibid.*, p. 31.
[11] *Ibid.*, p. 23.
[12] *Ibid.*, p. 38.
[13] *Ibid.*, p. 44. In referring to these lectures Berzelius confessed the following: "I believed, like all beginners, that completeness and thoroughness were the principal things.... The only one to derive any benefit from this was myself, since, as the number of my auditors diminished daily, I recognized that this manner of presentation was not the correct one." (*Ibid.*, p. 168, note 77.)
[14] Erroneously called "pharmacological" by Larsell.
[15] Söderbaum, J. J. Berzelius, "Autobiographical Notes," *loc. cit.*, pp. 66/67.

only through his lectures. It should not be overlooked that he participated to an extremely great extent in the preparation of the Swedish Pharmacopœias which appeared during the time of his active life. Most biographers have silently passed over the fact that the chemical part of the fifth edition of the Swedish Pharmacopoeia is the work of Berzelius. This edition, planned as early as 1793 but not completed until 1817 because of war and political unrest, offers not only an example as to the selection of drugs admitted, but its brevity and preciseness make it undoubtedly the best of all contemporary pharmacopoeias. In the preparation of the next (sixth) edition of this standard, published in 1845, Berzelius has likewise participated, this time as the chairman of a committee of revision especially appointed for this purpose.

"Berzelius has furthermore given vivid support to the establishment of an association of the Swedish pharmacists intended to promote pharmacy as a science. Having shown his appreciation of the beneficial effect of associations as early as 1807 by becoming one of the founders of the Swedish Society of Physicians, he helped in the founding of a similar pharmaceutical group. When in 1820 the newly formed Swedish Pharmaceutical Society under the chairmanship of Carl Göransson created a library and other useful institutions, Berzelius presented the young group with foreign periodicals and other substantial support besides helping by his advice.

"Naturally, more than through these local activities [in the interest of Swedish pharmacy] Berzelius has promoted pharmacy everywhere through his scientific life work and publications."[16]

It is of interest that the fifth edition of the Swedish Pharmacopoeia, the chemical part of which had been prepared by Berzelius, appeared in the same year in which the aforementioned German apothecary-chemist, Martin Heinrich Klaproth, passed away. It had been Klaproth who, together with the pharmacists Sigismund, Friedrich Hermbstädt (1760–1833) and Valentin Rose, Jr. (1762–1807), had made the new views of Lavoisier and the new chemical nomenclature the basis of the chemical part of the *Pharmacopoea Borussica* published in 1799, thus setting an example for his time which was followed everywhere. It deserves mention at this place that after the death of Klaproth "the chair of that prominent chemist and his position as counsellor in the *Obersanitätskollegium* in Berlin" was offered to Berzelius. He declined "this complimentary offer with thanks" since, as he expressed it, "I was indebted to my own native land for a passable and carefree living, with full opportunity to pursue the science to which I belonged...."[17]

Tributes to Berzelius.—The high esteem, the admiration, and the gratitude felt everywhere for the work and the personality of Berzelius expressed itself on the occasion of his death in numerous and detailed necrologies. But a closer examination shows that those published in English language pharmaceutical journals, for instance in the British *Pharm. J. & Trans.*, **8** 148(1848–1849) and in the *Amer. J. Pharm.* **21**, 189(1849), were reprints of a necrology published in *Lancet*, while the French *J. Pharm. Chim.* presented its readers with the reprint of a "Biographie Chimique" by P. Louyet. It is understood that these necrologies were of a general nature rather than expressing the particular feelings of the pharmaceutical world toward the great deceased.

It was the German pharmacists who conceived and performed a memorial to Berzelius which has to be regarded as one of the most beautiful tributes of gratitude ever offered to a great benefactor by those who consider themselves his beneficiaries. It is simultaneously proof of the wave of liberalism and universalism, expressing itself in the high emphasis placed on intellectual and humanitarian achievements, which swept over the Germany of 1848.

A "General German Pharmaceutical Congress" ("Deutscher Allgemeiner Apotheker Congress") convened at Leipzig on September 12 and 13, 1848, and its chairman, the pharmacist Dr. L. F. Bley, told in his opening address that "the year 1848 carrying with it the dawn of German liberty has been joyfully welcomed by the pharmacists" who hoped for redemption from "the pressure of tutelage and the domination by bureaucrats."[18]

It was on the second day of this Congress that the pharmacist Dr. E. Geffcken of Lübeck read an "Appeal to the German Pharmacists for the Establishment of a Memorial to Berzelius" from which the following significant part may be quoted:

"Berzelius' professorship of chemistry and pharmacy was not restricted to Sweden where the pharmaceutical profession owes primarily to him the dignified position recognized all over the other parts of Europe; he was, if not directly then indirectly, the teacher of all of us. For where in our fatherland can a pharmacist be found, and be it the most simple practitioner, who does not follow in the one way or another the theoretical or practical rules set by Berzelius. Alas, gentlemen, just here in Leipzig we are at a place from where, multiplied by the printing press, the products of Berzelius' mind have been distributed all over Germany, sometimes even earlier than in Sweden. In this respect it may suffice to point, as offering adequate evidence, to the five or six editions of his comprehensive textbook of chemistry."[19]

Geffcken suggested as a more fitting memorial to Berzelius, to be presented to the Swedish Academy of Science, a wreath of silver oak foliage with as many individual leaves in solid silver as pharmacists who had attended the 1848 Pharmaceutical Congress at Leipzig.

On August, 7, 1849, Geffcken reported that his appeal had been successful and his suggestion realized, and that "the firm Th. Strube and Son in Leipzig had produced a real piece of art." Having told that the wreath had, on December 11, 1848, been sent to the Swedish Academy of Science together with a poem from the pen of Emanuel Geibel,[20] Geffcken concluded as follows:

"The silver wreath was placed on a silver-trimmed black velvet pillow. In a case attached to it were: 1. the beautiful poem by Geibel printed with silver letters on black parchment; 2. the presentation

[16] See footnote 3.
[17] Söderbaum, J. J. Berzelius, "Autobiographical Notes," *loc. cit.*, p. 95.

[18] *Arch. Pharm.*, **106**, 83(1848).
[19] *Arch. Pharm.*, **106**, 117(1848).
[20] Emanuel Geibel (1815–1884) was one of the best-known representatives of German conventional poetry about the middle of the nineteenth century. He advocated the political unification of Germany under an emperor, not as a republic, hence was called the herald of the emperor ("Der Kaiserherold").

document. A letter expressing the gratitude of the Academy of Science at Stockholm has informed us that the wreath has been deposited beneath the picture of Berzelius in the room reserved for the sessions of the academy."[21]

The first paragraph of the letter of thanks mentioned above, and addressed to the "highly esteemed Pharmaceutical Congress at Leipzig," testifies to the respect in which German cultural and scientific achievements and standards were held at this time by the world at large. It reads as follows:

"The Swedish nation as well as all the other nations pursuing scientific aims are since days far remote indebted to Germany's noble and intelligent people for the unveiling of many a scientific truth and for a variety of useful inventions. Sometimes Sweden has had the satisfaction of repaying, in its limits, this debt. In more recent time it was Berzelius who has furnished his fatherland with such an opportunity. The Academy of which he was the most precious adornment, has received the most beautiful proof of appreciation of this fact from one of Germany's most reputed associations."

Geibel's poem was reprinted in three different modifications, in golden as well as silver letters on blue ground, and in black on white glossy paper. All three modifications, sold for the benefit of old meritorious pharmacy clerks, showed the picture of Berzelius enshrined by the wreath of silver oak leaves and followed by the poem which, in free English translation, appears below.

It is significant that the poem pays homage to Berzelius in the most general terms without mentioning pharmacy at all, except in the dedicatory caption. German pharmacy had, in this case, made itself the speaker for German science on the whole.

There cannot be any doubt. The great Swedish chemist and pharmacy have been connected in many and varied ways. It was not accidental happenings in the youth of Berzelius, some more or less ephemeral early "work in a pharmacy," that served as a kind of vantage point. It was the inescapable constellation of cogent circumstances which forced them together, the great chemist and the time-honored profession in which chemistry as a science has found an early place and continuous cultivation.

Pharmacy as an applied science owes Berzelius so much that it is entitled to claim him as one of its Patron Saints on the basis of one of the most elementary human rights: the right of the grateful to express his gratitude.

[21] *Arch. Pharm.*, **110**, 127(1849).

"TO THE MEMORY OF THE MASTER IN SCIENCE
J. J. v. BERZELIUS
THE GERMAN PHARMACISTS, 1848

For the immortal wreath on the slumbering head of Berzelius
Germany joins and presents gratefully leaves of her oaks
For him loved Mother Nature. Behold, her veil, the much folded
Smilingly threw she aside for him, her favorite son.
Graciously taught she him the secret of natural forces
Which she, ready to bless, calmly had kept in her breast.
Now, however, he solved, an enlightened disciple of Isis,
Thousands of riddles, unlocked numberless wonders to us.
As out of barren rock once Moses elicited water,
He out of metal and stone conjured up sources of life.
What, for the sake of mankind, helpful is joining in matter,
What is adversely opposed, what is harmoniously solved,
To the mortals he showed. Then death reluctantly touched him
Bringing the favorite son back to the mother again.
Sweden weepingly buries the dead, all Europe deplores him
But posterity's offerings are admiration and thanks."

CORRECTION

Add to the article entitled "A New Absorption Delaying Vehicle for Penicillin," by F. H. Buckwalter and H. L. Dickison, THIS JOURNAL, **37**, 472 (November, 1948) a footnote reading: "Received June 14, 1948, from the Research Laboratories, Bristol Laboratories Inc., Syracuse 1, New York."

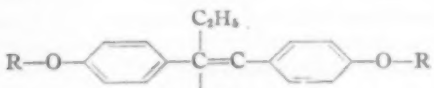
The Antibacterial Action of Basic Ethers of Diethylstilbestrol and 4,4′-Dihydroxydiphenylsulfone*

By D. E. SHAY, G. P. HAGER, L. RICE, and K. DAILEY

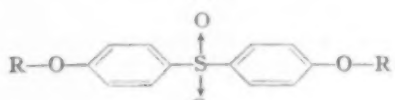
The preparation and the antibacterial activity *in vitro* of five substituted aminoalkyl ethers of diethylstilbestrol on *Staphylococcus aureus* (209), *Eberthella typhosa, Streptococcus pyogenes*, and *Mycobacterium tuberculosis* var. *hominis* and the results of *in vivo* studies of the compounds against *Mycobacterium tuberculosis* using guinea pigs are reported.

Three basic ethers of 4,4′-dihydroxydiphenylsulfone were prepared and tested *in vitro* against *Escherichia coli, Staphylococcus aureus* (209), and *Mycobacterium tuberculosis* var. *hominis* and were found to possess no antibacterial action.

THE diethylaminoethyl ethers of diethylstilbestrol and hexylresorcinol (stilbestryl-bis (2-diethylaminoethyl) ether and hexylresorcinol-bis (2-diethylaminoethyl) ether) have been shown to possess bactericidal action on cultures of *Mycobacterium tuberculosis* var. *hominis* at rather high dilutions (1). Before proceeding to *in vivo* studies using infected animals, it was decided to explore the possibility that the *in vitro* activity might be greater in homologous basic ethers of diethylstilbestrol (I), or in basic ethers of other phenols. It was of particular interest to include in this study basic ethers of 4,4′-dihydroxydiphenylsulfone (II), because of the known antitubercular activity of Promin (sodium 4,4′-diaminodiphenylsulfone-N,N′-didextrose sulfonate)



(I)



(II)

where R = substituted aminoalkyl group.

Attempts to prepare certain of the substituted aminoalkyl ethers by the procedure previously published (1) for preparation of stilbestryl-bis (2-diethylaminoethyl) ether resulted in polymerization of the substituted aminoalkyl chloride. The method was especially unsatisfactory when using 2-(4-morpholino) ethyl chloride which is known to polymerize readily in the methanol used as the solvent (2). It was possible to prepare stilbestryl-bis 2-(4-morpholino) ethyl ether when no polar solvent was used; and much better yields of all the stilbestryl ethers were obtained under these conditions. The disodium salt of diethylstilbestrol was conveniently prepared by reaction of diethylstilbestrol with sodium powdered in xylene. Better results were obtained, however, if the disodium salt was previously prepared by reaction of diethylstilbestrol with a solution of sodium ethoxide in alcohol followed by precipitation with ether and drying. This substance reacted readily with the basic alkyl halides when a mixture of the two in xylene was refluxed for a short time. The same procedure was quite satisfactory for preparation of certain basic ethers of 4,4′-dihydroxydiphenylsulfone.

Reports on the antibacterial properties of diethylstilbestrol and related compounds have appeared from time to time. Russ and Collins used diethylstilbestrol successfully in the treatment of twenty-five cases of prepuberal vulvovaginitis (3). The effectiveness in such infections might be due to the estrogenic activity of the compounds. Faulkner showed that diethylstilbestrol and 4-hydroxy-α,α'-diethylstilbene possess bactericidal activity when tested on *Mycobacterium tuberculosis in vitro* (5).

Substituted aminoalkyl ethers of other phenolic compounds have been tested for antitubercular activity. Some of the diethylaminoalkyl ethers of triiodophenol were found by Saz, Johnston, Burger, and Bernheim to be bactericidal in their action on human and bovine tubercle bacilli *in vitro* (4). It was found that oxygen uptake by the bacilli was decreased, growth *in vitro* inhibited, and the formation of tubercules in the omentum of infected guinea pigs reduced by several of the compounds tested.

Burger, Wilson, Brindley, and Bernheim synthesized several dialkylaminoalkyl ethers of iodinated phenols with variation in the structure of the ether chain (6). The ethers with a 2-methylpiperidinopropyl radical were highly effective on the tubercle bacilli. Activity and toxicity in guinea pigs were usually greater in dialkylaminopropyl than in the corresponding dialkylamino-

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ethyl ethers. On the other hand, shortening of the ethylene group as in a trihalogenophenoxydiethylaminomethane derivative, caused complete loss of antitubercular action.

Replacement of nuclear iodine by other halogens did not lead to a definite pattern correlating chemical structure to antitubercular activity. Nonhalogenated dialkylaminoalkyl phenyl ethers were found to lack antitubercular properties.

The recent investigations of Chapman, Hager, and Shay (1) revealed the antibacterial properties of stilbestryl-bis (diethylaminoethyl) ether dihydrochloride for *Mycobacterium tuberculosis* var. *hominis*, *Staphylococcus aureus* (209), and *Eberthella typhosa* when tested by a modified Food and Drug Administration procedure (8). The favorable results obtained by these workers was the stimulus for further studies with similar compounds.

The results of the *in vitro* antibacterial tests of five stilbestrol derivatives on *S. aureus* (209), *E. typhosa*, *Strept. pyogenes*, and *M. tuberculosis* var. *hominis* are reported. *In vivo* studies with *M. tuberculosis* var. *hominis* using guinea pigs are also included.

A preliminary investigation of basic ethers of 4,4′-dihydroxydiphenylsulfone was carried out in a manner similar to that used in the study of the stilbestryl ethers.

EXPERIMENTAL

Basic Ethers of Diethylstilbestrol.—A slight modification in the procedure previously reported (1) for the preparation of stilbestryl-bis(2-diethylaminoethyl) ether made possible the production of this compound in greater yields and was applicable to the preparation of other related compounds which could not be prepared satisfactorily by the original procedure.

The disodium salt (67 Gm., 0.25 mol.) of diethylstilbestrol was prepared by dissolving diethylstilbestrol in a solution of sodium (11.5 Gm., 0.5 equivalent) in absolute ethanol (250 ml.). After refluxing for thirty minutes, the disodium salt of diethylstilbestrol was obtained as a grayish white solid by removal of part of the alcohol by distillation under reduced pressure followed by precipitation with ether.

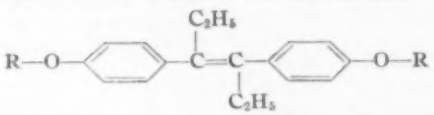
A mixture of the disodium salt of diethylstilbestrol (0.05 mol.), the substituted aminoalkyl chloride (0.2 mol.), and xylene (75 ml.) was refluxed for one to four hours until the suspension of fluffy amorphous solid had changed to a granular, crystalline precipitate of sodium chloride. The reaction mixture was cooled, diluted with an equal volume of ether and extracted with 10% hydrochloric acid. After washing the hydrochloric acid solutions with ether, the free base was precipitated by the addition of 20% sodium hydroxide solution and was then extracted with ether. The ether solution was washed with 20% sodium hydroxide solution to remove any phenolic substances and dried with anhydrous magnesium sulfate. The hydrochloride of the basic ether was prepared from the ether solution in the usual way and purified by repeated recrystallization from mixtures of absolute ethanol and ethyl acetate, ether or acetone.

The compounds prepared by this method are listed in Table I together with pertinent data.

Basic Ethers of 4,4′-Dihydroxydiphenylsulfone.—4,4′-Dihydroxydiphenylsulfone was prepared by treatment of phenol with fuming sulfuric acid (7). The disodium salt was prepared by dissolving 4,4′-dihydroxydiphenylsulfone (25 Gm., 0.1 mol.) in a solution of sodium ethoxide (0.2 mol.) in absolute ethanol (250 ml.). After heating on a steam bath for thirty minutes, treating with charcoal, filtering, and distilling off most of the alcohol under reduced pressure, ether was added and the precipitated white solid was filtered and dried.

A mixture of the disodium salt of 4,4′-dihydroxydiphenylsulfone (0.05 mol.), the substituted aminoalkyl chloride (0.15 mol.), and xylene (75 ml.) was refluxed for two hours. The free basic ethers were recovered from the reaction mixture as directed in the procedure for preparation of the stilbestryl ethers. The hydrochlorides could be prepared only as oils or amorphous solids. The picrates were the only salts that could be obtained in crystalline form, Table II.

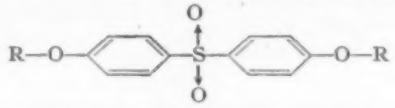
TABLE I.—BASIC ETHERS OF DIETHYLSTILBESTROL



R	M. P. of Dihydrochloride °C.	Yield, %	Formula	Analysis, %	
				Calcd.	Found
2-Diethylaminoethyl-	238–239	57	$C_{30}H_{48}Cl_2N_2O_2$	N, 5.19	5.05
2-Diethylaminopropyl-	(Oil)	..	$C_{44}H_{56}N_8O_{16}$[a]	N, 11.76	11.72
3-Diethylaminopropyl-	215–215.5	55	$C_{32}H_{52}Cl_2N_2O_2$	N, 4.93	4.73
2-(4-Morpholino)ethyl-[b]	255–256	40	$C_{30}H_{44}Cl_2N_2O_4$	N, 4.94	4.61
3-(2-Methylpiperidino) propyl-[c]	232–233.5	29	$C_{34}H_{56}Cl_2N_2O_2$	N, 4.52	4.20

[a] Picrate (m. p. 194–6°).
[b] Morpholine ethanol supplied by Carbide and Carbon Chemicals Corporation.
[c] 3-(2-Methylpiperidino)-1-propanol supplied by Eli Lilly and Company.

TABLE II.—BASIC ETHERS OF 4,4′-DIHYDROXYDIPHENYLSULFONE



R	M. P. of Picrate, °C.	Formula of Picrate	Analysis % Calcd.	Found
2-Diethylaminoethyl-	...	$C_{26}H_{42}N_8O_{19}S$	N, 12.35	12.17
3-Diethylaminopropyl-	165–169	$C_{26}H_{42}Cl_2N_2O_4S$[a]	N, 5.09	4.88
3-(2-Methylpiperidino) propyl-	206–207	$C_{42}H_{50}N_8O_{16}S$	N, 11.35	11.15

[a] Dihydrochloride.

BACTERIOLOGY

Stock solutions (1:100) for performing *in vitro* studies on *S. aureus*, *E. typhosa*, and *Strep. pyogenes* were prepared. From these solutions, dilutions (1:1000; 1:2500; 1:5000; 1:10,000; 1:20,000) of each compound were made. For each compound four sets of five tubes each containing 10 ml. of each of the above dilutions were prepared and sterilized in the autoclave.

For the *in vitro* studies on *M. tuberculosis*, two sets of five dilutions (1:10,000; 1:30,000; 1:50,000; 1:70,000; 1:100,000) of each compound were prepared.

Solutions for study of the *in vivo* antibacterial action of these compounds against *M. tuberculosis* were prepared as follows: 6 ml. of the 1:100 stock solutions of each compound was pipetted into large 250-ml. graduates and normal saline was added to give a total volume of 200 ml. These solutions were then transferred to 250-ml. Erlenmeyer flasks, resulting in a concentration of 150 μg. of each compound per 1 ml. of solution. To prepare a 300-μg. dose per 1 ml., 12 ml. of the 1:100 stock solutions of each compound was pipetted into large 250-ml. graduates and normal saline was added to give a total volume of 200 ml.; these solutions were then transferred to 250-ml. Erlenmeyer flasks, closed with rubber stoppers, and sterilized at fifteen pounds pressure (121°) for fifteen minutes.

The *p*H ranges of the various solutions as determined by means of the Beckman *p*H meter are given in Table III.

The culture medium selected for the tests on *S. aureus* and *E. typhosa* was nutrient agar. This was prepared according to the A. P. H. A. standard method. The medium selected for *Strep. pyogenes* was blood agar prepared in the usual manner. The medium selected for the *in vitro* studies of *M. tuberculosis* var. *hominis* was Petragnani's medium.

The organisms *S. aureus*, *E. typhosa*, and *Strep. pyogenes* were transferred on five successive days preceding the day on which they were to be used in the experiment.

IN VITRO TESTS

The *in vitro* test employed was a modified F. D. A. method of determining phenol coefficients. This modification consisted of a change in the time intervals from one, ten, and fifteen minutes to one, ten, and sixty minutes, respectively. Twenty-four hours before the tests were to be run, a standard 4-mm. loopful of a culture of each organism was transferred into separate sterile tubes containing 10 ml. of nutrient broth and incubated at 37°. After incubation for twenty-four hours bacterial cell counts were made. These results are given in Table III. To one set of dilutions of the compound, 0.5 ml. of the standardized broth culture was added. At intervals of one, ten, and sixty minutes, 1 ml. of each dilution of the compound containing the organism was plated. The plates were incubated at 37° for forty-eight hours. The results of the antibacterial tests are tabulated in Table III. This procedure was followed with each of the five compounds.

The tests on *Strep. pyogenes* were performed as above with the exception that no attempt was made to count the total number of colonies as had been done in the previous cases with *S. aureus* and *E. typhosa*. Instead, the plates were classified into two groups, according to the presence (+) or absence (—) of hemolysis. The results of this test are shown in Table III.

The *in vitro* test employed on *M. tuberculosis* was performed as explained previously for *S. aureus* and *E. typhosa*, with the exception of a slight change in inoculation of the medium. To one set of dilutions—1:10,000, 1:30,000, 1:50,000, 1:70,000, and 1:100,000—of each compound, 0.5 ml. of the treated positive tubercular sputum was added. After periods of one, ten, and sixty minutes, a standard 4-mm. loop of the organism compound mixture was streaked on sterile Petragnani's slants and incubated at 37° for six weeks. Results of these readings are recorded in Table III.

RESULTS

***In vitro* Results on *Staphylococcus aureus* 209.**—Table III includes all the results of the *in vitro* tests performed in the experiment. Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *S. aureus* (209) in a dilution of 1:2500 after a one-minute exposure and in a 1:5000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride shows antibacterial properties in a 1:2500 dilution after a one-minute exposure and in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride shows antibacterial properties in a 1:5000 dilution after a one-minute exposure and in a 1:10,000 dilution after a sixty-minute exposure. The two compounds, stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride and stilbestryl-bis (2-diethyl-amino-

propyl) ether (free base), show no evidence of possessing antibacterial properties in the five dilutions employed.

***In vitro* Results on *Eberthella typhosa*.**—Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *E. typhosa* in a 1:5000 dilution after a ten-minute exposure and in a 1:20,000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride is antibacterial to this organism in a 1:5000 dilution after a one-minute exposure and in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride is antibacterial to this organism in a 1:2500 dilution after a sixty-minute exposure. Stilbestryl-bis [3(2 methylpiperidino) propyl] ether dihydrochloride is antibacterial in a 1:5000 dilution after a one-minute exposure and in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis (2-diethylaminopropyl) ether (free base), show no evidence of possessing antibacterial properties in the five dilutions employed.

***In vitro* Results on *Streptococcus pyogenes*.**—Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *Strep. pyogenes* in a 1:10,000 dilution after a ten-minute exposure and in a 1:20,000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride is antibacterial in a 1:5000 dilution after a one-minute exposure and in a 1:20,000 dilution after a ten-minute exposure. Stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride is antibacterial in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride is antibacterial in a 1:10,000 dilution after a one-minute exposure and in a 1:10,000 dilution after a ten-minute exposure. Stilbestryl-bis (2-diethylaminopropyl) ether (free base) failed to show evidence of any antibacterial properties in the dilutions employed.

***In vitro* Results on *Mycobacterium tuberculosis* var. *hominis*.**—Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *M. tuberculosis* var. *hominis* in a 1:10,000 dilution after a ten-minute exposure and in a 1:50,000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride is antibacterial in a 1:30,000 dilution after a one-minute exposure.

TABLE III.—RESULTS OF *in vitro* STUDIES OF THE SEVERAL STILBESTROL DERIVATIVES ON BACTERIA

Compound	Organism	Antibacterial Dil. in 1000's	Activity Time
Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride; *p*H, 6.3–6.9	*S. aureus* 209[a]	2.5	1
		5	60
	E. typhosa[b]	5	10
		20	60
	Strep. pyogenes	10	10
		20	60
	M. tuberculosis (var. *hominis*)	10	10
		50	60
Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride; *p*H, 6.3–7.1	*S. aureus* 209	2.5	1
		10	60
	E. typhosa	5	1
		10	60
	Strep. pyogenes	5	1
		20	10
	M. tuberculosis (var. *hominis*)	30	1
Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride; *p*H, 5.6–6.6	*S. aureus* 209	5	1
		10	60
	E. typhosa	5	1
		10	60
	Strep. pyogenes	10	1
		20	10
	M. tuberculosis (var. *hominis*)	10	10
		100	60
Stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride; *p*H, 5.6–6.6	*S. aureus* 209	...	None
	E. typhosa	2.5	60
	Strep. pyogenes	10	60
	M. tuberculosis (var. *hominis*)	...	None
Stilbestryl-bis (2-diethylaminopropyl) ether (free base); *p*H, 6.4–7.0	*S. aureus* 209	...	None
	E. typhosa	...	None
	Strep. pyogenes	...	None
	M. tuberculosis (var. *hominis*)	10	10
		70	60

[a] One ml. of broth contained 12×10^9 organisms.
[b] One ml. of broth contained 9×10^9 organisms.

minute exposure. Stilbestryl-bis (2-diethylaminopropyl) ether (free base) shows complete absence of antibacterial properties in the five dilutions employed.

***In vitro* Results on *Streptococcus pyogenes*.**—Stilbestryl-bis (diethylaminoethyl) ether dihydrochloride is antibacterial to *Strep. pyogenes* in a 1:10,000 dilution after a ten-minute exposure and in a 1:20,000 dilution after a sixty-minute exposure. Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride is antibacterial in a 1:5000 dilution after a one-minute exposure and in a 1:20,000 dilution after a ten-minute exposure. Stilbestryl-bis [2(4-morpholino) ethyl] ether dihydrochloride is antibacterial in a 1:10,000 dilution after a sixty-minute exposure. Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride is antibacterial in a 1:10,000 dilution after a one-minute exposure and in a 1:100,000 dilution after a sixty-minute exposure. Stilbestryl-bis (2-diethylaminopropyl) ether (free base) is antibacterial in a 1:10,000 dilution after a ten-minute exposure and in a 1:70,000 dilution after a sixty-minute exposure. The compound, stilbestryl-bis [2(4-morpholino)ethyl] ether dihydrochloride, failed to show evidence of being antibacterial in the five dilutions employed.

The three basic ethers of 4,4′-dihydroxydiphenyl-

sulfone, Table II, were inactive *in vitro* against *E. typhosa*, *S. aureus* (209), and *M. tuberculosis* var. *hominis*.

IN VIVO TESTS

Three groups of ten guinea pigs each were numbered one to thirty inclusive and assigned to separate cages; group A—experimental (1–10); group B—drug control (11–20), and group C—organism control (21–30).

On the first day of the tests the weights of all animals were determined and recorded. The ten "experimental animals" and the ten "organism control animals" were injected subcutaneously with 0.5 ml. of a treated positive tubercular sputum. All of the "experimental animals" and all of the "drug control animals" were given their previously assigned doses of the stilbestrol derivatives intraperitoneally. These twenty animals continued to receive their respective doses of the stilbestrol derivatives in the same manner every forty-eight hours for a period of seven weeks. The weights of all animals were checked and recorded every seven days. When an animal died before the termination of the seven-week period, it was weighed and autopsied, and smears were taken of all organs which showed indications of tubercle lesions. The smears were stained by the Ziehl-Neelsen technique and examined for *M. tuberculosis*. At the end of the seven-week period all animals that had not died were weighed, killed, and autopsied, and smears were made of all organs which showed indication of tubercle lesions. These smears were also stained by means of the Ziehl-Neelsen method and examined for *M. tuberculosis*. Results of the *in vivo* studies are given in Table IV.

The "experimental animals," which received 0.5 ml. of treated positive sputum on the first day of the test and forty-eight-hour intraperitoneal injections of the stilbestrol derivatives for a period of seven weeks, showed on autopsy and staining, tubercle bacilli at all sites of injection of the organism and at least two organs. Animals numbered 6, 7, and 9 died on the 18th, 6th, and 26th days, respectively. All of the ten animals of the "drug control group," which received forty-eight-hour intraperitoneal injections of the stilbestrol derivatives for the seven-week period, survived. With the exception of animals numbered 13 and 14 the change in body weight over the seven-week period was negligible. Animals numbered 13 and 14, which received the 150 and 300 μg. doses, respectively, of stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride, showed

TABLE IV.—RESULTS OF THE *in vivo* STUDIES WITH M. TUBERCULOSIS VAR. HOMINIS[a]

Compound[b]	Animal Number	Dosage Every 48 Hrs. in μg.	Initial Wt., in Gm.	Wt. in Gm. at Autopsy	Day of Death and Autopsy	Location of Org. at Autopsy[c]	Wt. in Gm. on 49th Day of Test
I	1	150	570	427	49th	A, B, C, D, E	
	2	300	670	580	49th	A, B, C, D, E	
II	3	150	720	540	49th	A, D, E	
	4	300	720	617	49th	A, B, D	
III	5	150	520	360	49th	A, C, D	
	6	300	550	420	18th	A, B, D	
IV	7	150	600	450	6th	A, B, D	
	8	300	700	580	49th	A, B, C, D, E	
V	9	150	450	340	26th	A, B, D, F	
	10	300	500	407	49th	A, C, D, E	
I	11	150	450	...	...	...	440
	12	300	450	...	...	...	445
II	13	150	420	...	...	...	340
	14	300	450	...	...	...	360
III	15	150	450	...	...	...	497
	16	300	500	...	...	...	547
IV	17	150	400	...	...	...	417
	18	300	450	...	...	...	490
V	19	150	400	...	...	...	392
	20	300	520	...	...	...	542
	21	...	420	240	41st	A, D	
	22	...	400	360	49th	A, B, E	
	23	...	450	292	49th	A, B, D, E	
	24	...	370	285	49th	A, B, D, E	
	25	...	420	340	49th	A, B, D, E	
	26	...	400	392	49th	A, B, D, E	
	27	...	440	367	49th	A, B, D, E	
	28	...	520	480	49th	A, B, E	
	29	...	450	390	45th	A, E, F	
	30	...	450	270	33rd	A, B	

[a] Animals 1–10 represent the guinea pigs of the Experimental Group injected subcutaneously on the first day of the experiment with 0.5 ml. of positive treated tubercular sputum. Animals 11–20 Drug Control Group. Animals 21–30 represent the guinea pigs of the Organism Control Group injected subcutaneously on the first day of the experiment with 0.5 ml. of positive treated tubercular sputum.

[b] I—Stilbestryl-bis (2-diethylaminoethyl) ether dihydrochloride.
II—Stilbestryl-bis (3-diethylaminopropyl) ether dihydrochloride.
III—Stilbestryl-bis (2(4-morpholino) ethyl) ether dihydrochloride.
IV—Stilbestryl-bis (3(2-methylpiperidino) propyl) ether dihydrochloride.
V—Stilbestryl-bis (2-diethylaminopropyl) ether (free base).

[c] A—Site of injection of organisms, B—liver, C—spleen, D—lung, E—lymph gland, F—intestine.

a loss of body weight of 80 Gm. in the first case and 90 Gm. in the second.

All animals of the "organism control group," which received 0.5 ml. of treated positive sputum on the first day of the experiment, showed on autopsy and staining *M. tuberculosis* at all sites of injection of the organism and in at least one organ. A marked loss of weight was observed in these animals over the seven-week period. Animals numbered 21, 29, and 30 died on the 41st, 45th, and 33rd days, respectively.

SUMMARY

1. The preparation of a series of substituted aminoalkyl ethers of diethylstilbestrol and 4,4′-dihydroxydiphenyl sulfone is described.

2. Stilbestryl-bis [3(2-methylpiperidino) propyl] ether dihydrochloride was the most active of the group *in vitro* using *S. aureus*, *Strep. pyogenes*, and *M. tuberculosis* var. *hominis*. The 2-diethylaminoethyl ether of diethylstilbestrol was most active against *E. typhosa*. The basic ethers of 4,4′-dihydroxydiphenyl sulfone showed no activity in the *in vitro* tests.

3. The basic ethers of diethylstilbestrol were shown to be inactive when tested therapeutically in guinea pigs infected with *M. tuberculosis*. All uninfected animals which received forty-eight-hour injections of the basic ethers of diethylstilbestrol survived the seven-week observation period.

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Studies on the Pharmacology of *Euphorbia pilulifera**,†

By LLOYD W. HAZLETON and REBECCA C. HELLERMAN

Pharmacologic, antianaphylactic, and antihistaminic properties of *Euphorbia pilulifera* were investigated. Dealcoholized fluidextract of euphorbia was used except where otherwise noted. Oral doses of 30 cc./Kg. to mice and 15 cc./Kg. to guinea pigs were not acutely toxic. Rats fed 1.0 per cent and 5.0 per cent powdered euphorbia in the basic laboratory diet for ninety-seven days gained weight normally and showed no gross pathology on autopsy. Blood pressure and respiration studies in dogs showed that euphorbia administered intravenously resulted in a marked but fleeting vasodepression with little effect on respiration. Guinea pigs actively sensitized to egg white were protected from subsequent lethal anaphylactic shock by oral administration of euphorbia about one hour prior to injection of egg white. Euphorbia antagonized the action of histamine on isolated guinea-pig intestine.

INTRODUCTION

THE PLANT *Euphorbia pilulifera* is one of some 2500 members of the euphorbiaceæ family. In the National Formulary, Seventh Edition (1), *Euphorbia pilulifera* was designated as pill-bearing spurge but was deleted from the Eighth Edition. The drug of commerce consists of the dried plant. Many members of the euphorbiaceæ family are described as being relatively toxic and containing resins or latex and it is therefore important to distinguish the pilulifera from other members of its family in considering its pharmacology and therapeutic uses.

A review of textbook material (2–4) reflects the traditional use of *Euphorbia pilulifera* in the treatment of such conditions as asthma, hay fever, bronchitis, and other respiratory conditions. The synonyms of the plant, as well as of its preparations, further substantiate the observations that the drug has long been associated with the treatment of these disorders. In the latter part of the Nineteenth Century *Euphorbia pilulifera* (5, 6) received considerable clinical attention for its use in the treatment of asthma and a review of the literature will indicate that from the clinical standpoint it was considered to be quite effective. This popularity is reflected in the compound elixir of euphorbia, National Formulary, Seventh Edition (1), which carries the synonym of Hare's Elixir. In commerce this preparation was known as Hare's Anti-Asthmatic

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Elixir and is prepared from the fluidextract of *Euphorbia pilulifera*, also official in National Formulary, Seventh Edition.

Subsequent to the period referred to above little, if any, scientific work was conducted on *Euphorbia pilulifera*, but during this time the techniques and methodology of investigating allergic and asthmatic conditions underwent tremendous strides. Among the significant developments was the elucidation of the possible role of histamine as a factor in anaphylactic shock and allergies. Significant progress was made in the experimental studies of shock, which came to be known as foreign protein reaction and is typified by the lethal effects of injected egg white in guinea pigs following prior sensitization to this material. More recently exhaustive studies have been conducted on two groups of synthetic drugs, which are referred to generally as antihistaminic agents and antispasmodics.

In view of the long history of use, the earlier significant clinical studies and the lack of recent investigations taking advantage of the improved techniques available, it seemed desirable to re-evaluate the pharmacology of euphorbia on an experimental basis. In the report to follow, any reference to euphorbia designates *Euphorbia pilulifera*.

PREPARATIONS USED

The following preparations were used during the course of the studies:

1. Finely ground *Euphorbia pilulifera*, the pharmacognostic authenticity of which was established through the cooperation of Dr. Heber W. Youngken. This material *per se* was used in the subacute feeding experiments.

2. From the powdered euphorbia fluidextracts were prepared according to the National Formulary, Seventh Edition, using Process A, with diluted alcohol as the menstruum. Due to the alcohol content the fluidextract was not used directly in any of the studies.

3. From the fluidextract a dealcoholized fluidextract was prepared by evaporating a measured quantity of the fluidextract over a water bath to a syrupy consistency and making up to original volume with distilled water. This material was used for the determination of oral toxicity, for the investigation of smooth muscle activity and antiallergic properties, and is referred to in the text as dealcoholized fluidextract.

4. To clarify the dealcoholized fluidextract and to remove insoluble vegetable extractives, this material was filtered through Lloyd Reagent (a purified aluminum silicate). This material was used for the study of the pharmacological properties following intravenous administration and in some of the smooth muscle experiments, and is referred to as partially purified.

5. To investigate in a preliminary manner the stability of the active principles, portions of the above clarified solution were placed in containers immersed in boiling water for fifteen minutes, and checked for potency of smooth muscle activity.

6. Since it has been reported (2) that *Euphorbia pilulifera* might contain an unstable glucoside, the clarified solution was treated with *N*/10 hydrochloric acid, 1 cc. to 5 cc. of solution, maintained at the temperature of boiling water for fifteen minutes, then checked for potency of smooth muscle activity.

EXPERIMENTAL

Acute Toxicity.—Dealcoholized fluidextract of euphorbia was given to mice by oral administration (Table I). The dose was limited by the large volume, 75 cc./Kg. being as large as was practical to administer. There was very little effect except for slight depression and catharsis at the higher doses.

TABLE I.—ACUTE TOXICITY FOR MICE OF ORALLY ADMINISTERED DEALCOHOLIZED FLUIDEXTRACT OF EUPHORBIA

Dose, Cc./Kg.	Number of Mice	Living	Dead
5	2	2	0
10	2	2	0
20	2	2	0
30	10	10	0
60	10	8	2
75	10	10	0

Partially purified dealcoholized fluidextract of euphorbia, preparation 4, was given intravenously to mice (Table II). The injections were followed by very rapid respiration, marked vasodilation visible in the ears and tails, and occasional convulsions. One of the survivors at 10 cc./Kg. had hemorrhages into the eyeballs and sockets but no other gross pathology was observed. The LD_{50} is 7.4 cc./Kg. with an f of 1.28 (7).

TABLE II.—ACUTE TOXICITY FOR MICE OF INTRAVENOUSLY ADMINISTERED DEALCOHOLIZED FLUIDEXTRACT OF EUPHORBIA (FILTERED THROUGH LLOYD REAGENT)

Dose, Cc./Kg.	Number of Mice	Living	Dead[a]
5.0	12	9	3
7.5	10	5	5
10.0	14	4	10

[a] LD_{50} = 7.4 (f = 1.28).

Intravenous administration of lethal doses to rabbits resulted in marked respiratory stimulation followed by slowing and cessation while the heart was still beating.

Prior to tests for protective action against anaphylactic shock, oral doses of 0.5 cc./Kg. to 15 cc./Kg. were given to guinea pigs with no apparent toxicity.

Chronic Toxicity.—Three groups of 10 male albino rats each were used in chronic feeding experiments. The control group received laboratory diet only, the second group received laboratory diet containing 1% added euphorbia, while the third group received laboratory diet containing 5% of euphorbia. Each rat was individually housed and

had free access to food and water. Body weight and food consumption were determined weekly. One rat in the 5% level died after eighty-eight days from respiratory and middle ear infection. After ninety-seven days all remaining rats had gained weight normally (Table III). Following this period the rats were injected with 1 cc. egg white intraperitoneally (8). Euphorbia had no effect on the nonlethal hypersensitivity normally observed in the rat. The rats showed no gross pathology at autopsy.

Repetition of these experiments was made during March, April, and May, when the diet consisted primarily of dry pellets with only supplemental greens. A second lot of fluidextract of euphorbia prepared from the original lot of crude drug was used for these experiments. In contrast to the uniformity obtained in the first series, this second series showed marked irregularity, both in controls and treated groups. Of 16 animals, 3 proved to be totally immune to the administration of 3 or more

TABLE III.—SUMMARY OF FOOD CONSUMPTION FOR RATS ON SUBACUTE FEEDING OF EUPHORBIA FOR A PERIOD OF 97 DAYS[a]

Group	No. of Rats Start	Finish	Av. Weight Start	Finish	No. of Rat Days	Percentage of Theoretical	Total Food and Euphorbia Consumed, Gm.	Food Consumed — Total, Gm.	Av./ Rat/ Day, Gm.	Euphorbia Consumed — Total, Gm.	Av./ Rat/ Day, Gm.	Av. Total/ Rat, Gm.
Control	10	10	105	336	970	100	19,463	19,463	20.1	....	..	....
1% Euphorbia	10	10	106	329	970	100	20,228	20,025.72	20.6	202.28	0.21	20.37
5% Euphorbia	10	9	105	320	961	97.1	21,035	19,983.25	20.8	1051.7	1.09	105.73

[a] Two animals from the control group, 2 from the 1%, and 3 from the 5% levels died of respiratory infections during the first ten days. These were replaced with rats of the same stock and no allowance for this replacement is made in the total.

Guinea-Pig Egg White Sensitization.—Active sensitization was induced in guinea pigs (9) by intraperitoneal injection of 5 cc. of egg white, 25% in physiologically normal saline. After two or more weeks the injection of 0.75 cc. undiluted egg white resulted in death from anaphylactic shock. Death was preceded by coma and severe dyspnea, accompanied by audible gasping and convulsive movements.

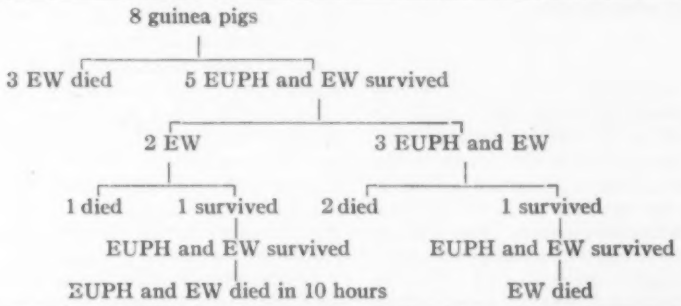
In an initial series of experiments, conducted during August and September, when the diet was primarily greens with pelleted food available, the oral administration of 15 cc./Kg. of dealcoholized fluidextract of euphorbia to sensitized guinea pigs one hour prior to intraperitoneal injection of egg white exhibited a marked antianaphylactic effect (Table IV). On the first test all three controls died in an average time of sixty-four minutes while five treated with euphorbia survived. Subsequent tests indicate that one of these might have been relatively immune to egg white, since it survived one dose unprotected. The most marked symptom of relief, aside from survival, was ease of respiration with total absence of audible gasping. Most of the animals became comatose for at least short intervals.

injections of egg white. The average time of death for 5 control animals was twenty-two minutes, while the average time for 8 treated animals was twenty-eight and a half minutes. This difference in survival time is not significant ($P = 0.2$). It should be noted that the survival time of the control animals approximates only one-third of the survival time of the control animals in Table IV, while the incidence of total immunity is marked. The group thus exhibited both unusual susceptibility and resistance to anaphylaxis in comparison to the initial studies.

At present it is impossible to evaluate properly this second series of experiments, since a new fluidextract, a change of diet, a different season, and an apparent extreme variability in response to administered egg white are factors to be considered. It is planned to repeat these studies to eliminate season and diet as possible variables.

To evaluate further the influence of euphorbia on respiration during egg white shock, several large sensitized guinea pigs were anesthetized with sodium pentobarbital and the respiration recorded by air tambour from the trachea. One guinea pig which served as a control had normal anesthetic respiration

TABLE IV.—INFLUENCE OF ORALLY ADMINISTERED DEALCOHOLIZED FLUIDEXTRACT OF EUPHORBIA ON LETHAL EGG WHITE ALLERGY IN ACTIVELY SENSITIZED GUINEA PIGS. EW = EGG WHITE; EUPH = EUPHORBIA GIVEN 1 HOUR IN ADVANCE OF EW



prior to the intraperitoneal injection of 0.75 cc. of egg white, after which the respiration became very poor and fluid formed in the tracheal canula. The respiration became slower and more shallow and ceased one-half hour after the injection of egg white. A second control animal had normal respiration before the injection of 0.75 cc. of egg white (Fig. 1).

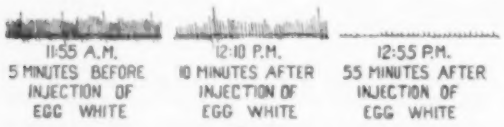

11:55 A.M.
5 MINUTES BEFORE INJECTION OF EGG WHITE
12:10 P.M.
10 MINUTES AFTER INJECTION OF EGG WHITE
12:55 P.M.
55 MINUTES AFTER INJECTION OF EGG WHITE

Fig. 1.—Respiratory tracings taken at intervals during egg white shock in unprotected guinea pig.

The respiration became slower and shallow fifteen minutes after the injection of the egg white. In one hour there was fluid in the tracheal canula, the respiration was very slow and shallow, and fifteen minutes later it stopped. A third guinea pig showing normal respiration was given 0.10 cc. of partially purified dealcoholized fluidextract of euphorbia intravenously. Following is an outline of the protocol describing the events shown in Fig. 2.

2:10—partially purified dealcoholized fluidextract of euphorbia, 0.10 cc. intravenously. Respiration more rapid.
2:15—0.75 cc. egg white intraperitoneally.
2:23—Respiration more shallow.
3:00—Respiration very shallow with some deep inspirations. 0.10 cc. euphorbia intravenously resulted in rapid respiration for about one minute.
3:33—0.10 cc. euphorbia intravenously. Respiration rapid for about one minute following injection, then it improved, was more regular, and there was an absence of the deep inspirations.
3:55—0.10 cc. euphorbia intravenously. Respiration rapid for about one minute. Respiration improved, looked similar to tracing before injection of egg white.

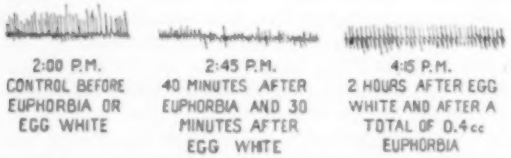

2:00 P.M.
CONTROL BEFORE EUPHORBIA OR EGG WHITE
2:45 P.M.
40 MINUTES AFTER EUPHORBIA AND 30 MINUTES AFTER EGG WHITE
4:15 P.M.
2 HOURS AFTER EGG WHITE AND AFTER A TOTAL OF 0.4 cc EUPHORBIA

Fig. 2.—Respiratory tracings taken at intervals during egg white shock in guinea pig protected by intravenous injections of euphorbia.

No more euphorbia was given and no more records were made, but the guinea pig lived until about 8:00 o'clock, which was almost six hours after the injection of egg white. During the entire course of the experiment there was no fluid in the tracheal canula as there had been in the controls. A fourth guinea pig was set up in the same manner. Respiration was normal when 0.75 cc. egg white was given intraperitoneally. Respiration became very shallow and irregular. The tracheal canula became filled with fluid. Thirty-seven minutes after the egg white, 0.075 cc. partially purified euphorbia was given intravenously. This dose had very little effect and 0.25 cc. partially purified euphorbia was given intravenously fifty minutes after the egg white. The animal died immediately after this dose, with no evidence of relief. Whether death was due to the large dose of euphorbia *per se* or was influenced by the advanced condition of shock could not be determined. It is interesting that the average survival time for this animal and the two controls, under anesthesia, was fifty-five minutes.

Blood Pressure and Respiration: Dogs.—In dogs under barbiturate anesthesia the intravenous injection of the partially purified fluidextract of euphorbia produces a characteristic vascular and respiratory sequence. The blood-pressure response is biphasic with an initial rise followed by a more pronounced fall. The pressor phase appears to be subject to tachyphylaxis since it may disappear after several doses. Figure 3 presents alternate injections of histamine and euphorbia, clearly indicating lack of antihistaminic action on the blood-pressure response. In general the depressor action of euphorbia resembles that of histamine, although the recovery in Fig. 3 (*d*) is more rapid than usual. The duration of action is brief and there is no evidence of accumulation.

Respiration was recorded by means of a Pfeiffer canula (10) using flutter valves. This maintains a constant minimum resistance to inhalation and expiration and therefore adds significance to these phases of the tracings. The respiratory effects appear to be primarily compensatory, and again the resemblance between histamine and euphorbia (Figs. 3 (*a*) and (*b*)) is marked. Following the initial dose of euphorbia, however, the nature of the respiratory response is markedly changed. While this change is apparent in the rate and duration it is more characteristically seen in the inspiratory phase as recorded by the air tambour from the trachea, which remains almost normal. Larger doses of euphorbia produce marked respiratory stimulation followed by apnea.

Euphorbia has no significant effect on the hemodynamic actions of epinephrine or acetylcholine and is not antagonized by doses of atropine which paralyze the parasympathetic receptor mechanism.

The following experiments were designed to determine whether the vasodepressor phase might be due to histamine or comparable substances in the plant extractive. A solution of histamine diphosphate was adjusted to *p*H 5.2 by the addition of potassium phosphate monobasic to bring it within the *p*H range of the partially purified fluidextract (*p*H 4.5). A portion of this solution was then filtered through Lloyd Reagent. In an anesthetized dog the buffered solution exhibited normal histamine activity while the Lloyd Reagent treated portion was totally devoid of activity. While this indicates that any histamine would have been removed by filtering the fluidextract through Lloyd Reagent, the dog was injected with 3 mg./Kg. of Tripelennamine hydrochloride (Pyrabenzamine HCl) intravenously. This dose almost completely antagonized the histamine but had no appreciable effect on the vasodepressor action of euphorbia.

Additional evidence of myotropic activity by euphorbia was obtained from two dogs severely

poisoned by O,O-diethyl-O-*p*-nitrophenyl thiophosphate (parathion), a cholinesterase inhibitor (11–13). Atropine administered intravenously to these dogs effectively antagonized the vagal stimulation and temporarily increased the respiratory exchange as recorded by the tambour. As respiratory exchange again decreased the intravenous administration of partially purified euphorbia produced its characteristic biphasic vascular response and increased respiratory exchange. Improvement occurred in both the inspiratory and expiratory phases, and with both atropine and euphorbia can probably be attributed to bronchial dilation, neurotropic and myotropic, respectively, since there is no change in rate.

Isolated Guinea-Pig Ileum.—Conventional techniques were employed in the study of isolated seg-

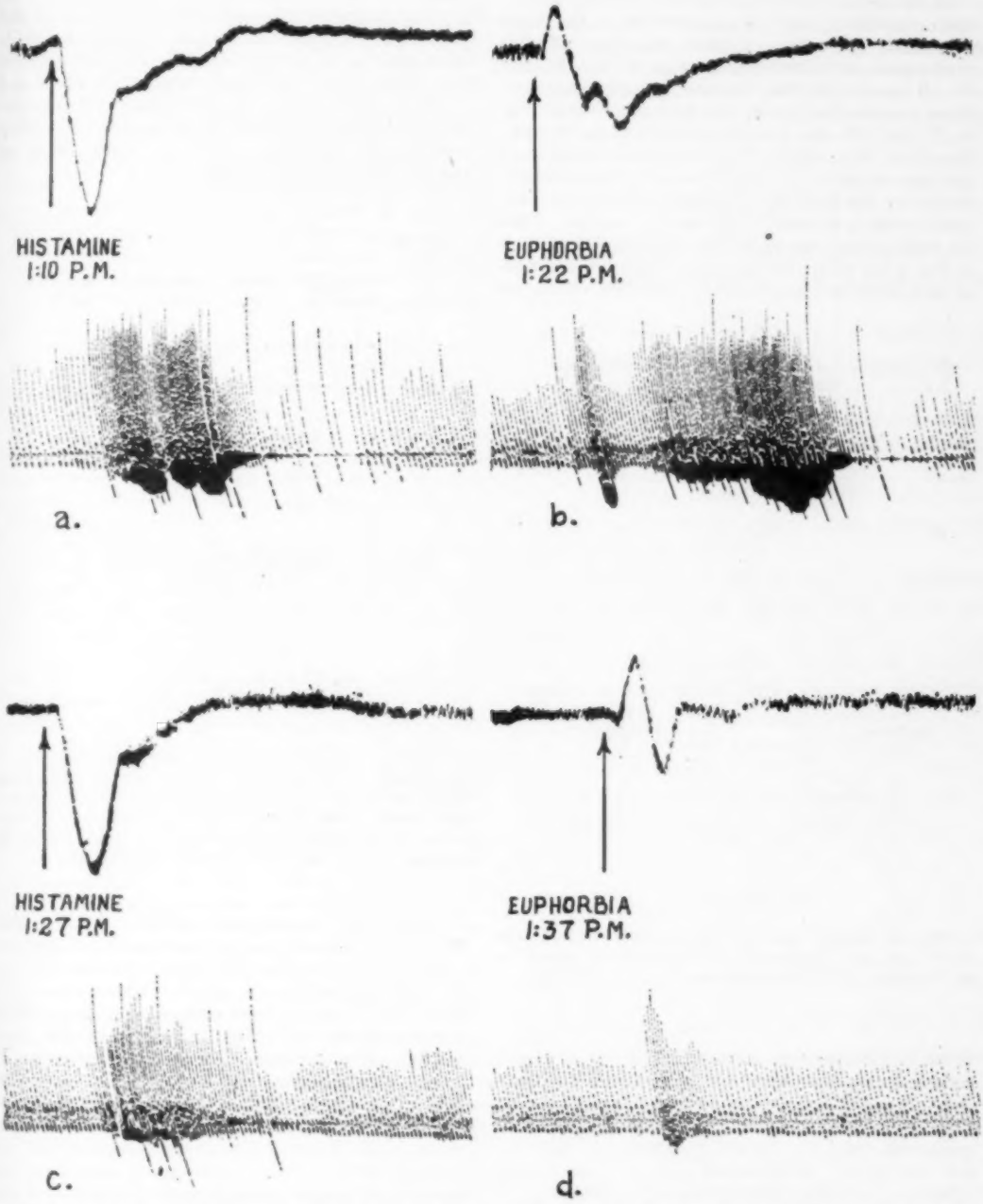


Fig. 3.—Alternate intravenous injections of histamine and euphorbia in dog weighing 10 Kg. under barbiturate anesthesia. Histamine was injected as the phosphate, 0.01 mg./Kg. Euphorbia was injected as the partially purified dealcoholized fluidextract, 0.1 cc./Kg. The top tracing is the blood pressure recorded from the carotid artery by mercury manometer. The bottom tracing is the respiration recorded from the trachea by air tambour.

ments of guinea-pig small intestine. The segments were placed in a chamber of 100-cc. capacity, stirred, and oxygenated by small bubbles of oxygen. The surrounding bath was maintained at 37° C. The lever was maintained at a ratio writing double the muscle excursion. Locke's solution was used exclusively in the investigations to be reported. Quantities of drugs quoted are those added to the chamber and therefore do not represent the final dilution in contact with the muscle.

In the early experiments the dealcholized fluidextract was filtered through gauze to remove the larger precipitate particles. Typical reactions of this preparation are illustrated in Figs. 4 (*a*), (*b*), and (*c*), all consecutive from the same intestinal segment. After standardization of the histamine dose (Fig. 4 (*a*)) the bath was washed and 1 cc. of the euphorbia added (Fig. 4 (*b*)). The sharp initial spike reaction appears to be characteristic of the drug as represented by the fluidextract. Repetition of the histamine now is without effect on the muscle. That the euphorbia is removable by washing is illustrated in Fig. 4 (*c*), where the response to the original dose of histamine is comparable. Complete relaxation

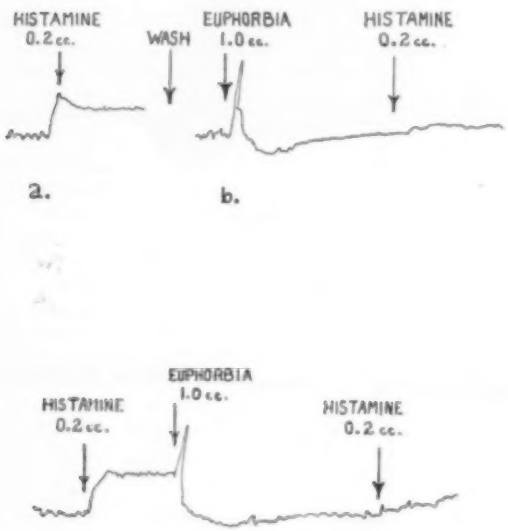


Fig. 4.—Relaxation and prevention of histamine (0.01 mg./cc.) contraction of guinea-pig ileum by euphorbia. (*a*, *b*, and *c* are consecutive tracings.)

of the histamine contraction, preceded by the characteristic spike, is achieved by the addition of euphorbia. A second dose of histamine at this point does not initiate a contraction. It should be noted that euphorbia did not relax the muscle appreciably below the normal baseline nor interfere with normal rhythmic activity. Dealcoholized fluidextracts of quassia, senna, and ergot were similarly tested for comparison. Quassia and senna exert no appreciable effect on the muscle and do not interfere with histamine contraction and its relaxation by euphorbia. On the other hand the ergot action is similar to euphorbia, producing a spike contraction and histamine antagonism without stopping rhythmic activity.

The activity of the total fluidextract of euphorbia was not diminished by filtration through Lloyd Reagent although there appears to be a marked diminution of the spike phase. Figures illustrating this on the ileum are omitted since the activity is retained after heating this preparation with acid (preparation 6) illustrated in Figs. 5 (*a*) and (*b*). Pertinent factors to be noted in Figs. 5 (*a*) and (*b*) are the ability of this preparation both to relax and prevent contraction from a relatively large dose of histamine in an active segment, the reduction of the spike phase, and the continuation of normal rhythmic activity following relaxation by euphorbia. In Fig. 5 (*c*) is illustrated the relaxation of a strong acetylcholine contraction, with no spike phase, by the partially purified product.

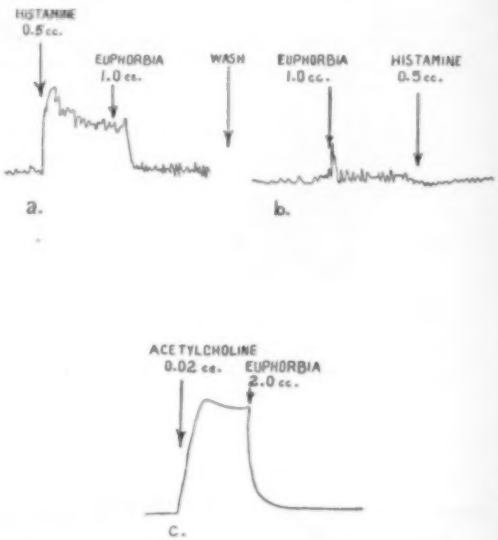


Fig. 5.—(*a* and *b*) Relaxation and prevention of histamine (0.01 mg./cc.) contraction of guinea-pig ileum by euphorbia filtered through Lloyd Reagent and heated with HCl in boiling water. (*c*) Relaxation of acetylcholine (0.1 mg./cc.) contraction by euphorbia filtered through Lloyd Reagent.

Preliminary experiments on differential extraction, not sufficiently complete to merit detailed discussion, indicate that (1) ether extraction of the dealcoholized fluidextract does not remove the active principle(s), the aqueous residue retaining most of the depressant and spike properties, (2) the ether portion, after removal of the ether, shows primarily stimulant properties, (3) an aqueous extract of euphorbia, representing 1 Gm./cc., contains about one-half the antihistaminic activity of the fluidextract, with proportional spike tendency, (4) an alcohol (95%) extract of the moist marc from the above aqueous extract, with the alcohol removed and replaced by water to represent 1 Gm. of the original drug per cc., contains almost entirely the stimulant or spike phase. The action closely resembles histamine, will reinforce a histamine contraction, and is antagonized by the dealcoholized fluidextract.

DISCUSSION AND SUMMARY

The empirical use and available literature on euphorbia suggest pharmacological investigations directed at an antihistaminic activity. Certain of the properties reported herein, such as the prevention or relaxation of histamine contractions of the guinea-pig ileum and a moderate degree of protection against egg white shock are suggestive of such action.

Certain other properties including relaxation of acetylcholine contraction, relaxation of bronchial constriction induced by chemicals other than histamine, and failure to counteract the vascular effects of injected histamine suggest that the action is not specifically antihistaminic. Supporting evidence is the absence of protection against egg white induced coma in guinea pigs and peripheral edema in rats.

Since euphorbia exerts no effect on the hemodynamic actions of epinephrine or acetylcholine and is not antagonized by atropine it may be assumed that the vascular actions are independent of the autonomic nervous system. The action is biphasic with only slight evidence of tachyphylaxis in the initial pressor phase and no evidence of accumulation.

The uniformity of these responses indicates a direct depressant action on smooth muscle rather than a specific antihistaminic or antiallergic activity. It is apparent after either oral or parenteral administration. This action is typically antispasmodic since doses which relax induced spasms of the ileum or bronchi have little effect on the normal tissue and do not interfere with normal rhythmic contractions of the ileum.

During the course of the investigations it became apparent that at least two active principles are present in *Euphorbia pilulifera*. The first of these caused a spike phase in guinea-pig ileum and was removed by filtration through Lloyd Reagent. In a review on histamine Gaddum (14) reports that Fuller's earth, magnesium trisilicate, and, under certain conditions, charcoal are capable of absorbing histamine and outlines certain conditions to be met for biologically identifying this material in mixtures. Although it was demonstrated that Lloyd Reagent does absorb histamine in the *p*H range of fluidextract of euphorbia, this cannot be taken as positive proof of its identity in euphorbia. The spike-producing principle can be partitioned into ether from the dealcoholized fluidextract, or extracted from the moist marc after aqueous extraction of the crude drug. It is therefore soluble in both water and organic solvents, perhaps more so in the latter.

The second principle appears to be the one responsible for the predominantly relaxing action on the various smooth muscles studied. It is water soluble, is not absorbed by Lloyd Reagent nor hydrolyzed by a mineral acid at the temperature of boiling water. It is not extracted from solution by ether in the acid *p*H range nor from the water-exhausted marc by alcohol. In aqueous solution the principle is stable over long periods of time at room temperature.

The acute and chronic toxicity of *Euphorbia pilulifera* is of an order which permits adequate dosage for pharmacological investigation with a substantial margin of safety. The mechanism of lethal action is not elucidated by the studies reported but it was observed that the principal symptoms of toxicity following intravenous injection are general depression and respiratory stimulation followed by depression and cessation. It is interesting to note that these observations coincide with those reported following oral administration to frogs, guinea pigs, and rabbits by Marsset in 1885 (6). This author also reported no observable direct effect on blood vessels, bronchi, alveoli, spinal cord, muscle (apparently skeletal), capillaries, or secretory glands. Within the limits of comparable techniques those observations are in general corroborated by the current investigations. The use of isolated tissue, intravenous administration, partial purification, histamine, and other pharmacological standards and the allergic phenomenon in the current studies serve to broaden appreciably the scope and elaborate these basic observations.

CONCLUSIONS

1. Fluidextract of *Euphorbia pilulifera* is relatively nontoxic following oral administration to mice.

2. The intravenous LD_{50} of the fluidextract, after filtering through Lloyd Reagent, is 7.4 cc./Kg. in mice.

3. The whole drug in levels up to 5% of the diet produced no toxicity in rats within ninety-seven days.

4. After oral administration the fluidextract exerts a prophylactic action against lethal egg white shock in guinea pigs.

5. Euphorbia appears to contain at least two active principles, the properties of which are discussed.

6. The antispasmodic action on smooth muscle predominates, with evidence presented that this mechanism is directly myotropic.

7. Since the antispasmodic principle is not adsorbed by Lloyd Reagent nor hydrolyzed by weak mineral acid, euphorbia does not appear to be alkaloidal or glycosidal in nature.

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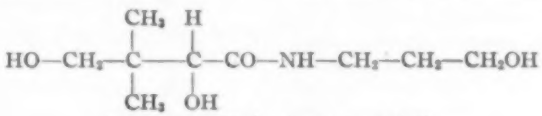
A Rapid Bioassay in Rats for Panthenol*

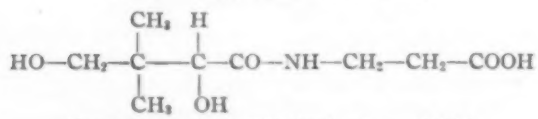
By L. DREKTER, R. DRUCKER, R. PANKOPF, J. SCHEINER, and S. H. RUBIN

An availability type of bioassay is presented for panthenol, the biologically active analog of pantothenic acid. The method is based upon the urinary excretion of pantothenic acid by the rat after dosage with panthenol. Pantothenic acid is determined by microbiological assay in the twenty-four hour urine. The specificity of the method has been demonstrated by comparison with rat curative and microbiological assays. The precision of the method, at suitable dosage levels, is of the order of 3 per cent in 8-rat assays.

In 1943 Pfaltz (1) showed that panthenol (I), the alcohol analog of pantothenic acid (II), is as effective a vitamin as the latter with regard to influence on growth and achromotrichia of black rats and alopecia in mice.



Panthenol (I) (mol. wt. = 205.3)



Pantothenic Acid (II) (mol. wt. = 219.2)

Burlet (2) later demonstrated that panthenol is oxidized by the rat and by man to pantothenic acid, as judged by urinary excretion of the latter. Burlet further found that, under certain conditions of dosage, the availability of panthenol to the rat and to humans exceeds that of calcium pantothenate, an observation which we (3) have recently confirmed and extended in humans.

Since recent trends (4, 5) in vitamin methodology have stressed the desirability of bioassays which measure *availability* to mammals, and because of the likelihood that panthenol is active by virtue of conversion to pantothenic acid, it was deemed desirable to evolve an assay method of the availability type. The method presented here depends upon measurement of the urinary pantothenic acid of rats after dosage with panthenol.

EXPERIMENTAL

Handling of Rats.—Male, albino rats weighing 150 Gm. or more were housed in pairs in metabolism cages. Improved cages, designed to give better separation of urine and feces, were used and will be described elsewhere (6). The rats were given distilled water and stock diet ("Friskie Dog Chow") *ad libitum*, the latter being ground and then wetted down with equal amounts of water to minimize spillage. After dosage with the panthenol solution, as described below, urines were collected for twenty-four hours in small, amber bottles under toluene. At the end of the collection period, the rats were removed from the cages, the lower screen containing the feces was removed, and the entire cage washed down (with the aid of a rubber policeman) with enough distilled water to bring the urine and washings to 1 liter.

Microbiological Assay.—Further dilutions were made as required for microbiological assay of the urinary pantothenic acid by the method of Skeggs and Wright (7), employing *Lactobacillus arabinosus*. The turbidity of the bacterial growth was measured in an Evelyn colorimeter after sixteen hours' incubation at 37° in a forced-draft incubator room.

It is not essential, though desirable, to analyze the urine from each cage separately. In the experiments described, up to 10 cages were used per sample and the urine from each was analyzed individually to provide data for statistical evaluation, presented below.

Basal Excretion.—In our panthenol studies on human subjects (3), it was the practice, as is commonly done (4, 5) in availability assays on humans, to determine the basal excretion of the vitamin prior to dosage. The necessity for such repetitive determination is contingent upon the relative magnitude of basal and dose and the variations in the two in relation to the desired precision of assay. On the basis of the data shown in Tables I and II and Fig. 1, it seems feasible to use a "statistical" basal value, which, in the present experiments, averaged 0.13 ± S.E. 0.01 mg. in rats that had grown from *ca.* 150 to *ca.* 465 Gm. (Table I). As determined by the method of Ives and Strong (8), the Friskie Dog Chow provided 9 γ per Gm. of Ca pantothenate; since these rats consumed 12–17 Gm. of the chow daily, an intake of *ca.* 0.1–0.15 mg. of pantothenic acid is indicated. The pantothenic acid requirement of rats of the age group used has been variously put at 0.025 mg. (9) to 0.1 mg. (10). Equating these estimates of intake, utilization, and urinary excretion leads to the conclusion that the basal excretions found are within a plausible range.

* Received June 7, 1948, from Nutrition Laboratories, Hoffmann-La Roche Inc., Nutley, N. J.

TABLE I.—LACK OF EFFECT OF REPEATED DOSAGE[a] WITH PANTHENOL UPON BASAL URINARY EXCRETION OF PANTOTHENIC ACID

No. of Doses Received[b]	Weeks Used	No. of Rats	Weight, Gm.	Urinary Ca Pantothenate, Mg./Day		
				Average	Range	Standard Error
0	0	54	150–180	0.13	0.08–0.17	0.01
5	5	60	275–320	0.13	0.08–0.19	0.03
18	18	20	388–465	0.15	0.12–0.17	0.01

[a] At intervals of one week.
[b] From 1 to 5 mg. of panthenol per dose by intramuscular injection.

It is further demonstrated in Table I that repeated dosage with panthenol over eighteen weeks at intervals of one week had no significant effect on the basal excretion. The interval of one week was chosen on the basis of control experiments in which it was found that the urinary excretion returns to the basal value within a week after dosage by injection or per os. Table II details a typical experiment, in which the excretion of pantothenic acid returned to basal levels by the fourth day after injection or feeding of a large dose of panthenol, 3.45 mg.

TABLE II.—DAILY URINARY EXCRETION OF PANTOTHENIC ACID AFTER A SINGLE LARGE DOSE OF PANTHENOL (3.45 MG.)[a]

Days after Dosage	Urinary Ca Pantothenate			
	Oral Dosage		Intramuscular Injection	
	Mg.	% of Dose	Mg.	% of Dose
Basal	0.12–0.17	3–4	0.12–0.17	3–4
1	2.36	59	2.56	64
2	0.38	9.5	0.34	8.5
3	0.24	6	0.22	5.5
4	0.21	5	0.19	5
5	0.17	4	0.17	4
6	0.14	3.5	0.18	4.5
7	0.14	3.5	0.15	4

[a] 3.45 mg. of panthenol = 3.45/0.86 = 4.0 mg. of Ca pantothenate.

These considerations provide grounds for the use of a "statistical" basal value, which in our experiments has averaged 0.13 mg. per day.

Route of Administration of Test Dose.—The data in Table II further show that, after a test dose of 3.45 mg. of panthenol, the excretion of pantothenic acid is essentially the same whether the dose be given by mouth or by intramuscular injection. Table III demonstrates that this identity of response persists through lower levels of the standard dose-response curve given in Fig. 1 (v.i.). Both methods of dosage therefore provide the same sensitivity of response. However, as a matter of practical convenience, we prefer intramuscular dosage, since injection (into the sacrospinalis and multifidis muscles) is more easily done routinely than oral dosage.

Standard Dose-Response Curve.—In addition to the statistical basal value described above, it was deemed feasible similarly to construct a statistical (standard) dose-response curve, and so lessen the routine labor of this bioassay. Figure 1 summarizes 54 trials of panthenol by intramuscular injection in 108 rats of doses up to 5.1 mg. The curve is essentially linear at doses above 1 mg. The equation for this line, calculated by the method of least squares, is $y = -0.16 + 0.73x$; the standard error of the estimate ($Sy{\cdot}x$), is 0.17 mg. (11). For the individual means shown in Fig. 1, the coefficient of variation, $s/\bar{x}$, ranges from 1.4% at a dose of 5.1 mg. to 7% at a dose of 1 mg. This linear portion of the curve is therefore quite suitable for the assay. The curvilinear portion below 1 mg. is much less suitable and would entail considerably greater errors.

Specificity of Assay

Non-effect of Pantoic Acid and Pantoyl Lactone.—There is little reason to believe that these split products of panthenol would interfere in the sense that these could be resynthesized in the rat to pantothenic acid, except for some data of Sarett (12) wherein, after feeding of pantoyl lactone or pantoic acid to humans, the pantothenic acid excretion increased from 3.5 to 6 mg. per day. In the present experiments (Table IV), the intramuscular injection of these split products, either alone or in combination with panthenol, had no effect upon the estima-

TABLE III.—COMPARISON OF ORAL AND INTRAMUSCULAR ADMINISTRATION OF PANTHENOL IN RATS

Dose		Urinary Ca Pantothenate	
Panthenol, Mg.	Ca Pantothenate Equivalent, Mg.	Oral, Mg./Day	Intramuscular, Mg./Day
3.45	4.0	2.36	2.56
1.72	2.0	1.25	1.21
0.43	0.50	0.40	0.35

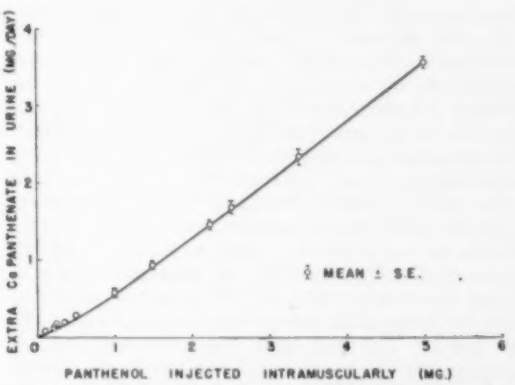


Fig. 1.—Relation between dosage with panthenol and urinary excretion of extra calcium pantothenate.

tion of the latter. Where panthenol was administered (expts. 3, 4, and 5, Table IV), the recoveries ranged from 92% to 96%.

TABLE IV.—NONEFFECT OF PANTOIC ACID AND PANTOYL LACTONE ON EXCRETION BIOASSAY OF PANTHENOL

No.	Injected	Panthenol Recovered Mg.[a]	%
1	3.5 mg. pantoic acid	0	..
2	3.3 mg. pantoyl lactone	0	..
3	2.5 mg. panthenol + 1.8 mg. pantoic acid	2.4	96
4	2.5 mg. panthenol + 1.7 mg. pantoyl lactone	2.3	92
5	2.5 mg. panthenol + 0.9 mg. pantoic acid + 0.9 mg. pantoyl lactone	2.4	96

[a] Corrected for basal excretion of 0.13 mg. per day.

Agreement with Curative Assays in Pantothenic-Deficient Rats.—The basic proof for specificity lies in comparisons with classical bioassays, wherein the vitamin deficiency is produced and cured. The chief limitation of such proof is the poorer precision of the curative bioassay. The comparison, nevertheless, provides a useful measure of specificity. For the curative assays, the diet used was essentially that of Henderson, *et al.* (10) with the following differences: 3% of cod-liver oil was substituted for 3% of the corn oil; biotin (2 μg.), *p*-aminobenzoic acid (0.25 mg.), inositol (0.5 mg.), and folic acid (2 μg.) were added to the vitamin supplement of Henderson, *et al.*, in the daily doses shown. The assays were conducted at two levels which, for the standard substance—calcium pantothenate—were 30 and 60 μg. per day; 10 rats were used at each level.

It was first necessary to ascertain the efficiency of panthenol in curing pantothenic deficiency in rats, relative to calcium pantothenate. Pfaltz (1) had previously demonstrated that these two substances have approximately equivalent activity in pantothenic deficiency. The present series of curative bioassays, based on growth responses, show that the activity is equivalent within the limits of error (v.i.) of the procedure, confirming Pfaltz' original observations. A typical set of response curves is detailed in Fig. 2.

The comparisons between the curative and excretion bioassays were carried out on ampuled solutions of panthenol which had been buffered in McIlvaine's phosphate-citrate buffer at graded *p*H from 3 to 8, preserved with methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate, and aged at 45° for periods up to sixty-three days. The results (Table V) expressed as a percentage value (excretion assay over curative assay) average 103% with a standard error of 2.7%, for the 15 samples tested. This agreement is eminently satisfactory, considering that two bioassays are involved. Since, as estimated by the method of Gridgeman (13), the standard error of a *single* curative bioassay was 8%, it is not surprising that the limits of the ratios observed range from 79% to 119%. The fact that the excretion method averaged 3% higher than the curative is not significant; *t* for the difference is 0.7; a *t* of 2.145 would denote significance at the 5% level.

Agreement with Microbiological Assays.—A further check on the specificity and precision of the excretion bioassay has been obtained by comparison

TABLE V.—COMPARISON BETWEEN CURATIVE AND EXCRETION METHODS FOR THE DETERMINATION OF PANTHENOL

Test Solution	Panthenol Found—Curative Assay, Mg./Cc.	Panthenol Found—Excretion Assay, Mg./Cc.	Excretion/Curative × 100, %
Initial solution	3.3	3.0	91
*p*H 3, 28 Days at 45°	2.3	2.5	109
*p*H 3, 42 Days at 45°	2.0	2.1	105
*p*H 3, 63 Days at 45°	1.8	2.0	111
*p*H 4, 28 Days at 45°	2.7	3.0	111
*p*H 4, 63 Days at 45°	3.0	2.8	93
*p*H 5, 28 Days at 45°	3.0	2.8	93
*p*H 5, 63 Days at 45°	2.9	2.9	100
*p*H 6, 28 Days at 45°	2.6	3.1	119
*p*H 6, 63 Days at 45°	2.9	2.9	100
*p*H 7, 28 Days at 45°	2.7	2.7	100
*p*H 7, 63 Days at 45°	2.3	2.6	113
*p*H 8, 28 Days at 45°	2.1	2.3	110
*p*H 8, 42 Days at 45°	2.4	1.9	79
*p*H 8, 63 Days at 45°	1.6	1.7	106
		Average = 103 ± (S. E.) 2.7	

with a microbiological assay method recently reported from this laboratory (14). Table VI gives typical comparative data by both methods for a vigorously aged multivitamin preparation. In order to cause partial decomposition, this solution was adjusted to *p*H 2 and heated at 45° for the periods shown in Table VI. This drastic treatment was necessary because of the slow rate of breakdown of panthenol under normal storage conditions. Satisfactory agreement between the two assay

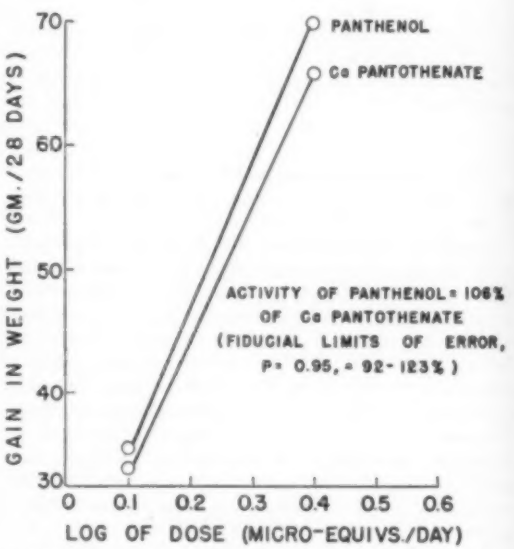


Fig. 2.—Relative activity in pantothenic-acid deficient rats.

methods was obtained for each solution, the average ratio being 102 ± 1.3% (S. E.). The deviation from 100% is not significant.

TABLE VI.—COMPARISON BETWEEN MICROBIOLOGICAL AND EXCRETION METHODS FOR THE DETERMINATION OF PANTHENOL

Test Solution,[a] Days at 45°	Panthenol Found: Microbiological Assay, Mg./Cc.	Panthenol Found: Excretion Bioassay, Mg./Cc.	Excretion: Microbiol. × 100, %
0	3.8	3.7	97
1	3.5	3.6	103
2	3.3	3.4	103
3	3.0	3.0	100
5	2.7	2.8	104
8	2.5	2.5	100
12	2.2	2.2	100
16	2.0	2.0	100
20	1.8	2.0	111
	Average = 102 ± 1.3 (S. E.)		

[a] The test solution was a multivitamin preparation which was adjusted to pH 2 and aged at 45° in order to promote more rapid breakdown of panthenol, with a view, especially toward formation of pantoyl lactone.

Precision of the Excretion Bioassay.—As a measure of the working precision of the method, it has been useful to calculate the standard error of the excretion assays on these aged panthenol solutions. These were 4-cage assays, i.e., 8 rats per assay, at working levels of 1–3 mg. per rat. Urine from each cage was analyzed separately. For the 15 samples involved, the coefficient of variation, $s/\bar{x}$, ranged from 1% to 5.4%, with an average of 3.2%. Errors of similar magnitude were found when the method was applied to various pharmaceutical preparations, both solid and liquid. As noted above, the working errors are considerably greater at doses below 1 mg. It is therefore desirable that solutions for intramuscular injection contain at least 1 mg. of panthenol per cc.

Example.—The following example illustrates the procedure used in this laboratory to assay panthenol in a liquid multivitamin preparation with a claim of 5 mg. per cc. Into each of 8 rats, kept in pairs in the metabolism cages, 1 cc. of the sample was injected. The twenty-four hour urine and washings from each cage were diluted to 1 liter and subdiluted to the proper concentration for the microbiological assay for calcium pantothenate. The Wood method (15) was used for calculating the results of the microbiological assays. The excretion values for the paired rats were 7.2, 7.4, 7.6, and 7.3 mg.; the average excretion per rat was therefore 3.69 mg. Correction for the "statistical" basal excretion of 0.13 mg. (v.s.) gave 3.56 mg. as the extra excretion due to the dose. When this figure was substituted for y in the formula for the dose-response curve, $y = -0.16 + 0.73x$, a potency of 5.1 mg. of panthenol per cc. was obtained. The standard error for this particular assay was calculated to be ±0.07 mg.

SUMMARY

An availability type of bioassay is presented for the determination of panthenol, the biologically active analog of pantothenic acid. The method is based upon the urinary excretion of pantothenic acid by the rat after dosage with panthenol. Pantothenic acid is determined microbiologically in the twenty-four hour urine. Specificity of the method has been demonstrated by comparison with rat curative and microbiological assays. Pantoic acid and pantoyl lactone do not interfere.

The dose-response relationship is essentially constant for rats varying in weight from 150 to 450 Gm. The usual working range (1–5 mg.) is sufficiently high to warrant the use of a statistically determined basal excretion. On a one-week dosage schedule, repeated administration of panthenol had no significant effect upon the level of basal excretion. The need for running concurrent standards or pre-dosage basal excretion has thus been eliminated.

The precision of the method can be judged by the fact that the average coefficient of variation $s/\bar{x}$, for 4-cage assays (8 rats) is 3 per cent.

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The Comparative Stability of Pantothenic Acid and Panthenol*

By SAUL H. RUBIN†

In vivo **studies have shown that panthenol, the alcohol analog of pantothenic acid, displays the vitamin activity, qualitatively and quantitatively, of pantothenic acid. Under certain conditions of dosage, the physiological availability of panthenol is superior to that of pantothenic acid. The present *in vitro* experiments demonstrate that panthenol is considerably more stable than pantothenate in acid solutions at *p*H 3 to 5.**

It is well known that the *p*H-stability pattern of pantothenic acid differs considerably from that of the other commonly used water-soluble vitamins which are sensitive to alterations in *p*H. Thiamine, riboflavin, and ascorbic acid are relatively stable in distinctly acid solution, become increasingly unstable as the reaction approaches neutrality, and are definitely unstable in alkaline solution. Pantothenic acid is most stable at *p*H *ca.* 6, increasingly unstable as the *p*H veers away to either side of 6 (1, 2). A detailed example of these opposing *p*H-stability relationships has been provided by Frost and McIntire (3), who determined the kinetics of hydrolysis of thiamine and pantothenate in acid solutions.

The practical difficulties inherent in this situation become sharply manifest in preparing *liquid* multivitamin preparations so as to provide maximum stability of the various components. The problem is more acute with regard to parenteral preparations because the permissible operating range of *p*H is somewhat circumscribed by the factor of pain on injection (4).

A plausible solution to this problem has been found in the pantothenic analog, panthenol, whose biological behavior is outlined in the preceding paper (5). Panthenol is more readily available than pantothenic acid when fed to the rat (5) and to man (6, 7) a circumstance which suggested greater stability of the former. This premise has been borne out by the comparative trials described in this report.

EXPERIMENTAL

Comparative stability trials were carried out both in pure solution—with added buffer and preservative—and in various multivitamin preparations.

Stability in Pure Solution.—Solutions of panthenol and sodium pantothenate at concentrations of 3 to 4 mg. per cc. were prepared in McIlvaine phosphate-citrate buffers (*ca.* 0.05 *M*) at graded *p*H from 3 to 8, and preserved with methyl *p*-hydroxybenzoate (0.02%) and propyl *p*-hydroxybenzoate (0.18%). The solutions were sealed in 5-cc. ampuls and kept in randomized distribution in a forced-draft incubator at 45 ± 0.5°. The use of phosphate-citrate buffer provided the *same* buffer anions at all *p*H, the only variable being the relative activities of buffer anions at different *p*H. This variable is not important in the present experiments, however, since the prime objective was to compare relative stability of the two compounds in pairs at equal *p*H.

The panthenol ampuls were assayed by the two bioassay methods described in the preceding paper (5), the curative and the excretion, and, in some cases, by a microbiological assay (8). Sodium pantothenate was determined by the curative bioassay, and by the microbiological procedure of Skeggs and Wright (9) with *Lactobacillus arabinosus*.

The results are summarized in Fig. 1. In order to segregate the results obtained by the curative bioassay, which was used for both panthenol and pantothenate, from the results by the other methods, the

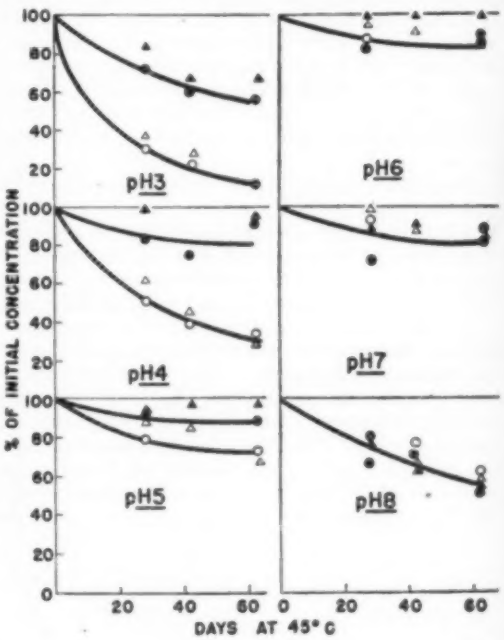

Fig. 1.—Comparative stability: panthenol and sodium pantothenate.

● Panthenol, curative bioassay in rats.
▼ Panthenol, excretion bioassay in rats.
○ Na Pantothenate, curative bioassay in rats.
△ Na Pantothenate, microbioassay.

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† The author is indebted to Dr. M. P. Furter for advice and encouragement in this study, and to Dr. A. Motchane for discussions of certain aspects of the problem.

stability curves have been drawn through the former. Inspection of Fig. 1 shows clearly, however, that, irrespective of the assay method, there are considerable differences in the relative stability of panthenol and sodium pantothenate, panthenol being distinctly superior in the more acid solutions. The better stability of panthenol is most manifest at *p*H 3 and 4; at *p*H 5, the advantage is relatively slight, while at *p*H 6, 7, and 8, there are no apparent significant differences between the two.

The *p*H values shown in Fig. 1 are those of the solutions initially. After aging at 45° for 63 days, the panthenol ampuls showed little or no change in *p*H, while the more acid solutions of sodium pantothenate (*p*H 3, 4) became somewhat more alkaline, as detailed in Table I. This tendency to an alkaline shift is associated with the greater breakdown of pantothenic acid and the release of the amino group of β-alanine. It is apparent that this shift would favor the stability of pantothenic acid to the extent that the *p*H approaches 6, the *p*H of optimum stability. Obviously, however, it exerted no decisive effect on the relative panthenol-pantothenic picture in the present experiments.

TABLE I.—CHANGES IN *p*H OF PANTHENOL AND SODIUM PANTOTHENATE SOLUTIONS
*p*H determined with a Beckman glass electrode meter

Panthenol		Sodium Pantothenate	
Initial *p*H	*p*H after 63 Days at 45°	Initial *p*H	*p*H after 63 Days at 45°
3.0	3.1	3.0	3.5
4.0	4.0	4.0	4.3
5.1	5.2	5.1	5.2
6.2	6.2	6.2	6.2
7.0	7.0	7.0	7.0
7.8	7.6	7.8	7.6

	Assay	% Loss
Panthenol, pure solution	Excretion bioassay	3
Panthenol, hexavitamin solution	Excretion bioassay	5
Na pantothenate, pure solution	Microbiological	69
Na pantothenate, hexavitamin solution	Microbiological	76

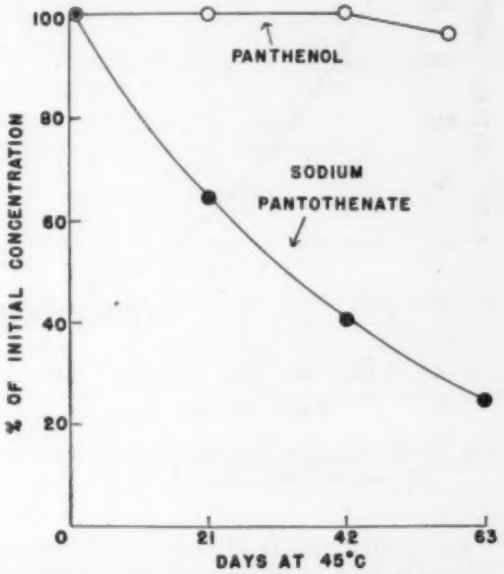


Fig. 2.—Relative stability of panthenol and sodium pantothenate in a liquid multivitamin preparation at *p*H 4.

Stability in Multivitamin Solution.—One of the more difficult stability problems presented by pantothenic acid occurs in liquid multivitamin preparations, which are usually made at *p*H *ca.* 4. Therefore, as a prime test of the comparative stability, an experimental hexavitamin preparation was studied containing thiamine, riboflavin, niacinamide, pyridoxine, ascorbic acid, and either panthenol or sodium pantothenate in equivalent amounts (*ca.* 5 mg./cc.). These were also aged at 45°. In these experiments, panthenol was determined by the excretion bioassay only, since, as has been demonstrated in the preceding paper (5) and in the experiments in pure solution detailed above, the two bioassay methods give essentially comparable results; the excretion bioassay is more precise, however. Sodium pantothenate was determined by microbiological assay with *Lactobacillus arabinosus*.

Figure 2 shows that the advantage of panthenol over pantothenate was maintained in this hexavitamin preparation, the loss of panthenol being nil up to forty-two days and possibly 5% at sixty-three days. The following tabulation of the values at sixty-three days at 45° delineates the close correspondence between relative stability in pure solution (Fig. 1) and in the hexavitamin preparation (Fig. 2); the presence of the other vitamins obviously did not alter the relative stability of panthenol or pantothenate.

Conversely, it was found that the other vitamins present—thiamine, riboflavin, niacinamide, pyridoxine, ascorbic acid—manifested the same stability irrespective of whether the preparation contained panthenol or sodium pantothenate.

The relative stability of panthenol and sodium pantothenate at *p*H 4 has also been studied at 60°. Figure 3 shows the results of a test of pure solutions at this temperature. Panthenol was determined by the excretion bioassay. Figure 3 represents essentially the same relative stability pattern as at 45°, and is further useful in showing that no critical temperature intervenes between 45° and 60°.

DISCUSSION

It is generally accepted that the first step in the breakdown of pantothenic acid is the splitting of the amide linkage between the lactone and β-alanine moieties. The work of Frost (2) on optical rotation as a measure of the stability of pantothenic acid supports this viewpoint. Frost and McIntire (3) showed that hydrolysis of pantothenate in acid solution follows a first-order reaction with respect to pantothenate concentration. Hydrolysis of pantothenate is therefore a less complicated process than, for example, the destruction of thiamine, which is subject to many influences other than *p*H, e.g., effect of buffer (10).

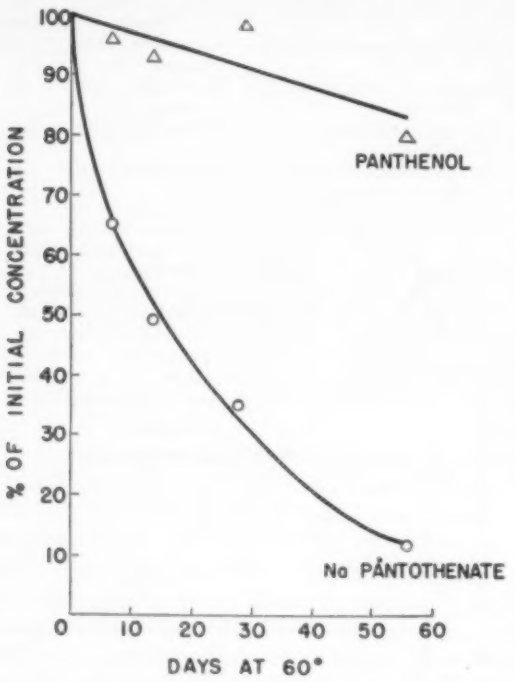
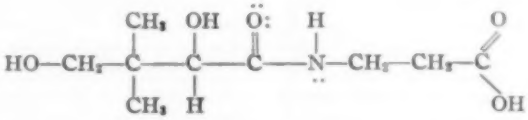


Fig. 3.—Comparative stability of Na pantothenate and panthenol at *p*H 4 and 60° C.

The greater stability of panthenol over pantothenate in acid solution appears to be associated with the lesser polarity of the terminal hydroxyl of panthenol as compared with the terminal carboxyl of pantothenic acid, and the relative influence of these on ease of hydrolysis of the amide linkage. The electronic configuration of the amide group in pantothenic acid may be formulated as follows:



It is well known that the first step in hydrolysis involves the addition of OH^- at the carbonyl carbon (transition state). This addition destroys the amide resonance. Conversely, weakening of the amide resonance makes the addition, i.e., hydrolysis, easier. Accordingly, the hydrolysis of amides is faster in acid solution than in alkaline (cf. 11).

The inductive effect of the terminal carboxyl of pantothenic acid, carrying a larger dipole than the terminal hydroxyl of panthenol, exerts a similar effect of increasing the positive charge on the nitrogen and thus weakening the resonance of the amide. The effect is particularly prominent in weak acid solutions where it is added to the effect of ammonium ion formation. (The amide being a very weak base, the ionization of N is not complete in the range of *p*H 3–4.) In alkaline solution (e.g., *p*H 8, Fig. 1), the resonance in the carboxyl group weakens the inductive effect on the nitrogen, and the rates of hydrolysis of pantothenic acid and panthenol become very nearly equal.

The better stability of panthenol in acid solution may also be involved in its superior physiological availability to rats and to humans (6, 7). The availability of pantothenic acid is diminished when taken with food (7, 12), whereas the availability of panthenol is not significantly affected (7). Silber (12) has shown in dogs that this effect on pantothenic acid is due, in part, to loss in the feces. However, a considerable portion of the pantothenic acid ingested is not accounted for. This unaccounted fraction may well be lost due to the greater acidity of the stomach after ingestion of food and consequent increased destruction of pantothenic acid.

The advantages of panthenol for multivitamin preparations are clearly apparent.. Not only does panthenol provide greater stability in the commonly used range of acid *p*H, but, moreover, it becomes possible to drop the *p*H somewhat so as to provide a better measure of stability for thiamine, ascorbic acid, etc., with relatively little sacrifice of panthenol stability.

SUMMARY

The comparative stability of pantothenic acid and panthenol has been evaluated in buffered solutions over the *p*H range 3 to 8 and in multivitamin preparations. As determined by two independent methods for each vitamin, including a rat curative bioassay in each case, panthenol is considerably more stable at *p*H 3–4, somewhat more stable at *p*H 5, and about the same as pantothenate at *p*H 6–8. In a hexavitamin preparation at *p*H 4, the relative stability was the same as in pure solution at the same *p*H.

The implications of these data are discussed in relation to mechanism, vitamin formulation, and to the physiological availability of these vitamins.

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A Modification of the U. S. P. Method of Assay for Belladonna Extract*

By A. S. RIDOLFO† and E. P. GUTH‡

A new technique for the assay of extracted material is proposed which substitutes the use of sintered glass funnels for the Soxhlet apparatus in removing the alkaloids from the extract. This procedure is proposed as it was found that the thimbles used in the assay were adsorbing substances which reacted positively to Valser's Reagent.

A NEW technique for the assay of belladonna extract is proposed which substitutes the use of sintered glass funnels for the Soxhlet apparatus. Compared with the official method, this procedure features ease of operation, saving of time, and a high degree of precision and accuracy. Sixteen different samples of Extract of Belladonna were examined, both by the U. S. P. method and by a modified method, which indicated that the proposed method is advantageous.

It is recommended that this procedure be considered for the estimation of the alkaloidal content of extracts. It is recognized that the official assay for the alkaloidal content of solanaceous drugs presents difficulties. Fricke and Kaufman (1) investigated the effect of the quality of chloroform and the type of starch used in the extract as problems in the assay for Extract of Beladonna. Durret (2) suggested the use of neutralized alcohol in the final evaporation of the chloroformic solutions to retard alkaloidal decomposition during assaying. Others (3) have shown that keeping the alkaloids in an acid *p*H would increase racemization and hydrolysis.

Although precautions were taken in this work to follow the suggested remedies, erratic results were still encountered in assaying the Extract of Belladonna. Upon investigation of possible reasons for these discrepancies, the discovery was made that the Soxhlet thimbles were adsorbing substances which reacted positively to Valser's Reagent. Even after continuous percolation of fifteen hours according to the U. S. P. directions, acid washings of the thimble gave a milkywhite precipitate with Valser's Reagent. This prompted the possible use of the sintered glass funnel as a substitute for the Soxhlet apparatus.

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DESCRIPTION OF APPARATUS

Equipment necessary for one unit:

1. A 250-ml. suction flask
2. A 50-ml. sintered glass funnel
3. A 50-ml. test tube
4. Porcelain crucible lid
5. A 5-ml. beaker
6. Suction tubing and screw clamps
7. Source of vacuum

The test tube is inserted into a 250-ml. suction flask, which is stoppered with a one-hole rubber stopper. The stem of the sintered glass funnel is extended through the stopper and into the test tube to a depth of about one inch below the side arm of the suction flask.

Any number of units may be attached to each other in a series by means of T-tubes. Clamps are so arranged that suction may be applied or removed from each unit. This is an important consideration for it permits the removal of the collected filtrates when necessary without disturbing the maceration or exhaustion of another unit and it permits the insertion of the 5-ml. beaker into the mouth of the test tube to receive the 1-ml. samples to test for the alkaloids (Fig. 1).

PROCEDURE

Approximately 3 Gm. of the accurately weighed extract is intimately mixed with two times its weight of washed sea sand (No. 20 mesh) and placed into the sintered glass funnel. Eighteen milliliters of the U. S. P. XIII alkaloidal extraction solution for solanaceous drugs is added and allowed to macerate for thirty minutes, keeping the funnel covered in order to prevent evaporation of the solvent. Slight suction is then applied by means of an aspirator and the contents of the funnel are sucked dry. The suction is then released and the collected filtrate is transferred from the test tube into a separatory funnel. This procedure is repeated four times using 10 ml. of ether and shortening the period of maceration to five minutes for each treatment.

Tests on 1-ml. samples of the filtrate with Valser's Reagent demonstrated the reliability of this method in exhausting the alkaloids.

The residue in the funnel is then washed with several 10-ml. portions of approximately $N/5$ H_2SO_4 until 1 ml. of the filtrate last collected is negative to Valser's Reagent. At this point the residue in the funnel should consist mostly of sand and starch with some extraneous matter. The acid washings are transferred to the separatory funnel containing the ether extracts and are used to extract the alkaloids from the ethereal solution. The remainder of the assay follows the official procedure

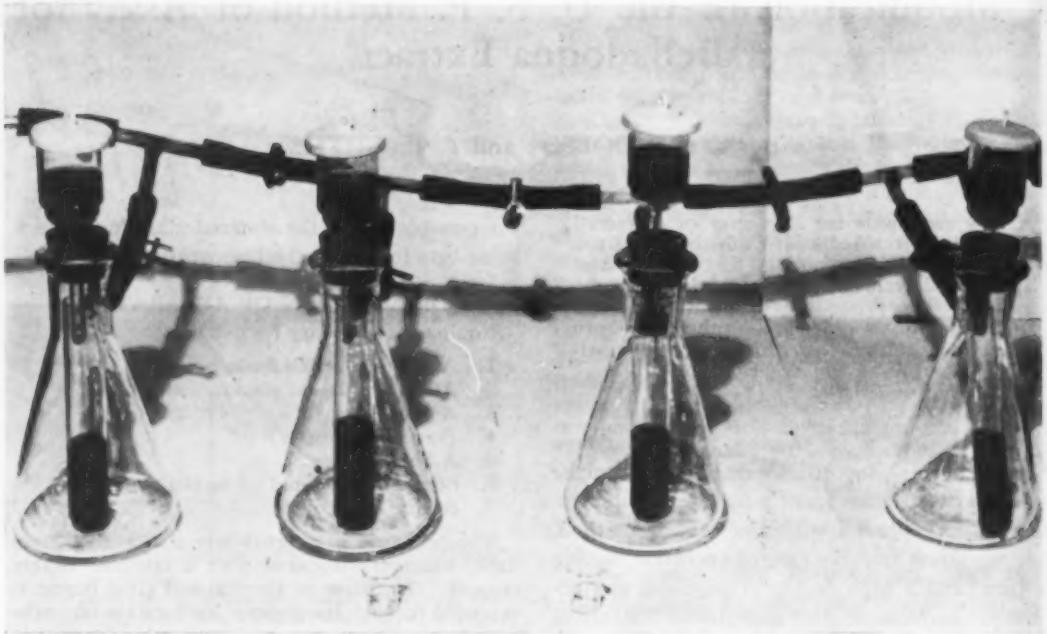

Figure 1.

except that it was found unnecessary to carry out the triple evaporation with chloroform as outlined in the official method. Instead a few milliliters of neutral alcohol was added at this point and the collected chloroform solutions were evaporated to dryness.

DISCUSSION

The results obtained and presented in Table I indicate the reliability of the substitution of a sintered glass funnel as a means of extracting the alkaloids of Belladonna from its extract. The use of a Soxhlet apparatus in the assaying of extracts is thought to be unnecessary since we are dealing with extracellular soluble material evaporated to dryness. By simple maceration and expression by suction, small quantities of solvent were found suitable to exhaust completely the alkaloids from the extract.

The method proposed also eliminates the necessity for maceration overnight. This period of maceration is probably needed in assaying the crude drug, but since extracts of belladonna represent the active constituents removed from the drug in a concentrated form, overnight maceration is not necessary, especially since the alkaloids of belladonna are readily soluble in alcohol, ether, or chloroform.

From a study of the values presented, it may be seen that the use of a sintered glass funnel in the manner outlined produced higher results when compared to the U. S. P. method. This may be accounted for by the completeness of the extraction of the alkaloids, and also by the smaller quantities of solvent necessary for extraction.

TABLE I.—ASSAY OF EXTRACT OF BELLADONNA BY U. S. P. AND MODIFIED METHOD[a]

Sample	U. S. P. Method	% Diff.[b]	Modified Assay	% Diff.
1	1.50	3.8	1.5	1.7
2	1.14	1.0	1.15	...
3	0.63	7.1	1.03	0.9
4	0.96	2.5	1.07	0.3
5	0.97	7.2	1.08	0.7
6	0.52	9.2	0.87	3.3
7	1.50	3.8	1.50	3.8
8	1.05	4.2	1.12	0.2
9	1.42	2.7	1.5	1.3
10	1.16	3.2	1.18	0.05
11	1.30	5.0	1.36	0.07
12	0.68	8.4	0.89	0.6
13	1.31	3.0	1.35	0.9
14	0.98	7.0	1.04	1.0
15	1.49	4.2	1.50	1.1
16	0.98	7.0	1.05	0.8

[a] Represents average of three assays for each sample.
[b] % difference is calculated on the highest and lowest values obtained for each sample.

SUMMARY

1. The substitution of a sintered glass funnel for the Soxhlet apparatus was found to be more efficient in the assay of Belladonna extract.

2. The time necessary to perform the assay is reduced to less than two hours.

3. Higher results are usually obtained.

4. Results are more uniform than those obtained by the U. S. P. method.

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Polarographic Determination of Folic Acid and Zinc Salts in Pharmaceuticals*

By JEANNE BUNDY DUNCAN and JOHN E. CHRISTIAN†

The quantitative estimation of zinc salts and folic acid by the polarographic method of analysis was investigated and the method was found to be applicable to pharmaceutical preparations. Barbituric acid was also studied, showing no oxidation or reduction at the dropping mercury cathode.

"POLAROGRAPHY" was developed as an analytical tool by Heyrovsky (1, 2) about twenty-five years ago. This electrochemical technique has been adapted in the ensuing years to almost every chemical and physical procedure. In certain cases, the simultaneous qualitative detection and quantitative determination of a number of different substances are possible from a single polarogram. The method is applicable over a range of 10^{-6} to 10^{-2} molarity, and best results are obtained at 10^{-4} to 10^{-3} molarity.

The basic concept of polarography lies in the principle that the electrolysis of an electrolyte in a cell activated by an easily polarizable dropping mercury electrode and a second large nonpolarizable electrode results in a current-voltage curve from which it is possible to determine both the nature and concentration of the reducible or oxidizable substance present.

Theoretically, any substance which is electroreducible or electrooxidizable within the potential range of the dropping mercury electrode can be analyzed by the polarographic method. In actual practice, this theory does not always hold true; nevertheless, the diverse applications of this procedure are of a wide variety.

A survey of the available literature reveals that the polarograph has not been used extensively in pharmaceutical analysis, but the possibilities in this field are almost unlimited.

EXPERIMENTAL

Apparatus and Methods.—This work was carried out with the use of the Sargent Model XX Heyrovsky Visible Recording Polarograph. The intersection point method of measuring wave height (diffusion current) as given by Müller (3) was used throughout the work. A capillary was used which had a $m^{2/3}t^{1/6}$ value of 1.684. Dissolved oxygen was removed from all solutions by bubbling hydrogen gas through the sample for at least five minutes. A mercury pool on the bottom of the electrolysis vessel served as the large nonpolarizable electrode.

Zinc Salts.—There are many references made to the determination of the zinc ion in the literature; however, there is no record of the application of the polarographic method to pharmaceutically important zinc salts.

Standard quantitative curves for several zinc salts were determined. Zinc chloride and zinc acetate of the N. F. VIII, in addition to zinc sulfate, zinc oxide, and zinc stearate, which are official in the U. S. P. XIII, were used. A solution of 0.1 molar ammonium acetate at its original *p*H of 6.75 was used as the supporting electrolyte. No maximum suppressor was required.

Samples were prepared for producing standard curves of the zinc salts by diluting the required volume of a 0.05 *N* solution of the salt with 0.1 molar ammonium acetate solution.

Since zinc chloride is very hygroscopic, it was dried at 110° for twenty-four hours before weighing. This procedure converts the $ZnCl_2$ to Zn_3OCl_2. Zinc oxide was treated with the minimum amount of diluted HCl necessary to cause solution before preparing the sample by the general procedure. Zinc stearate was placed in a crucible, and the fatty acids were burned off in an electric muffle furnace. The residual zinc oxide was then dissolved as described above.

These solutions were electrolyzed at a temperature of 25° with the sensitivity setting on the polarograph of 2–98 and a damping control of 3. The Initial Potential was 0 volt, and the Span Potential was 2 volts.

Table I shows the diffusion currents obtained for these solutions.

TABLE I.—AVERAGE[a] I_d OF ZN SALTS[b]

Concn.	Zn_3OCl_2	$ZnSO_4$	Zn Acetate	ZnO	Zn Stearate
0.00100 *N*	16.75[c]	18.86	18.10	17.44	15.90
0.00075 *N*	12.37	14.28	13.64	12.85	11.92
0.00050 *N*	8.52	9.79	9.43	8.79	8.96
0.00025 *N*	4.38	4.93	5.00	4.54	4.57
0.00010 *N*	1.90	2.06	2.01	1.81	1.75

[a] Average of from six to twelve determinations.
[b] In 0.1 *M* ammonium acetate.
[c] I_d expressed in microamp.

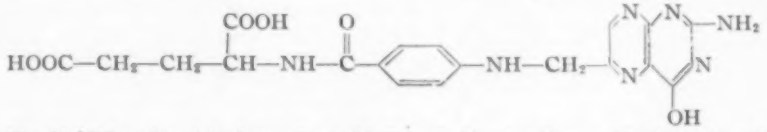
The average wave heights for each concentration shown in Table I when plotted on a graph as ordinate against concentration as abscissa produce a straight line, which can be used as a standard curve for determining unknowns of the same zinc salt.

Barbituric Acid.—The polarographic analysis of barbituric acid would seem dubious, since the literature indicates that the parent compounds do not react at the dropping mercury electrode. This ex-

* Received April 12, 1948, from the School of Pharmacy, Purdue University, Lafayette, Ind.
† The authors express their appreciation to the Lederle Laboratories division of the American Cyanamid Company for supplying the samples of folic acid.

perimentation revealed that barbituric acid gave no polarographic wave in supporting electrolytes of 0.1 molar ammonium chloride, 0.2 molar tetraethylammonium bromide, or in buffers ranging from *p*H 3 to 10.

Folic Acid.—One of the newer members of the vitamin B Complex group, folic acid, has recently been synthesized. The accepted structure for the Liver *L. casei* Factor or Folic Acid is (4):



N—[4-{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}benzoyl]-glutamic acid
Pteroylglutamic Acid

The above molecule shows several groupings which might lend themselves to polarographic analysis.

At the present time, a microbiological assay is used for the determination of folic acid (5).

A sample of 87.7% pure folic acid was obtained for experimentation. An unbuffered solution made by dissolving folic acid in 10% ammonium hydroxide with a supporting electrolyte of 0.1 molar ammonium chloride was electrolyzed with no results.

Buffer solutions were next prepared according to Kirkpatrick (6) and the U. S. P. (7). The concentration of the salts in the buffer solutions was great enough so that the buffer would also act as the supporting electrolyte.

Samples were prepared by treating the folic acid with the minimum amount of 10% ammonium hydroxide necessary to cause solution, and diluting with water to make a standard solution of 0.005 molarity. To the correct volume of folic acid standard solution was added 50 ml. of buffer, and the entire solution was then made up to 100 ml. with distilled water.

It was found that folic acid was not soluble in glacial acetic acid in the concentration necessary for polarographic analysis. Therefore, the alkaline buffers are much more efficient in the already alkaline solution of folic acid.

A preliminary investigation revealed that folic acid in buffered solutions of *p*H range of 3–10 gives a polarographic step. It was discovered that solutions having a *p*H of 6.5 or lower produce two polarographic waves; the first wave is usually proportional to concentration. The *p*H range of 8–10 was found to give the best results. Therefore, a series of folic acid solutions buffered to a *p*H of 9 with a buffer containing boric acid, potassium chloride, and sodium hydroxide (7) was selected to determine whether wave height (diffusion current) was directly proportional to the concentration of folic acid present in the solution. The diffusion currents expressed in microamperes resulting from these solutions are listed below.

Concentration	Microamperes
0.0001 *M*	2.18
0.00025 *M*	5.55
0.0005 *M*	10.90
0.00075 *M*	16.24
0.001 *M*	21.60

Each figure is an average of approximately seven different determinations. These solutions were electrolyzed at a temperature of 25° with a sensitivity setting on the polarograph of 2–95 and a damping control of 3. The initial potential was 0 volt, and the span potential was 3 volts.

These diffusion currents when plotted on a graph as ordinate against concentration as abscissa give a straight line slope. We can assume that under these experimental conditions the diffusion current of folic acid is a linear function of concentration.

The fact must be kept in mind that this standard curve was prepared using folic acid of 87.7% purity.

A typical polarogram of folic acid is shown in Fig. 1. This is a current-voltage curve of 0.00075 molar folic acid in a solution buffered to *p*H 9.

The half-wave value of the total applied E. M. F. for folic acid in a buffered solution of *p*H 9 appears to be approximately −0.87 volt. This half-wave potential seems to be independent of concentration.

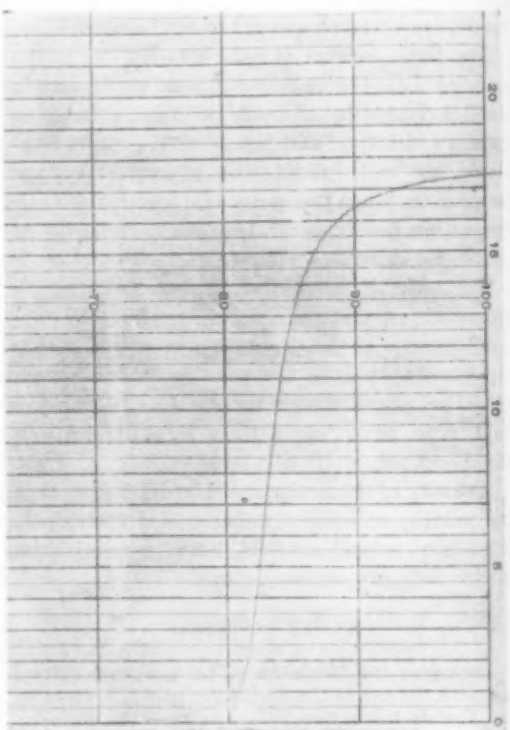


Fig. 1.—Polarographic wave of 0.00075 *M* folic acid.

The half-wave value does become slightly more negative with increasing *p*H, but the increase is not great enough to show that hydrogen ions are used or given off in the electrode reaction.

Solutions of folic acid buffered to a *p*H of 10 gave a wave with a maximum-like characteristic

which resulted in an irregular limiting current quite difficult to measure with accuracy. This maxima was eliminated in solutions containing only 0.002% gelatin, but the resultant wave heights are not proportional to concentration. Methyl red had no suppressive effect on this maxima. Addition of ions such as potassium, barium, and calcium were not helpful in obtaining a more regular wave. Where ions with higher valences were added, the half-wave potential seemed to be slightly more negative.

The procedure was also applied to a limited number of commercial tablets containing folic acid with good results.

SUMMARY

Various substances of pharmaceutical importance were investigated for the applicability of their determination by means of the polarographic method. Quantitative analysis was stressed in all cases.

Zinc chloride, zinc sulfate, zinc acetate, zinc oxide, and zinc stearate can be quantitatively determined by polarographic analysis in a supporting electrolyte of 0.1 molar ammonium acetate in concentrations ranging from 10^{-3} to 10^{-4} molarity.

The possibility of reducing the time required for barbiturate analysis, if the polarographic method could be applied to barbituric acid and its derivatives, was investigated. The experimentation revealed that barbituric acid gave no polarographic wave in supporting electrolytes of 0.1 molar ammonium chloride, 0.2 molar tetraethylammonium bromide, or in buffers ranging from *p*H 3 to 10.

The conditions under which folic acid can be quantitatively determined by the polarographic method have been described in detail. Best results were obtained when the aqueous solution of folic acid was buffered to a *p*H of 9 with a buffer solution containing boric acid, potassium chloride, and sodium hydroxide. The resultant half-wave value of the total applied E. M. F. is approximately −0.87 volt. Under these experimental conditions, the polarographic method was shown to give reproducible results for folic acid present in concentrations ranging from 10^{-3} to 10^{-4} molarity. This assay was also applied to a limited number of tablets containing folic acid with good results. The possibility of the application of this quantitative polarographic procedure to folic acid preparations is apparent.

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Urine Iodine Following Ingestion of Different Iodine Preparations*

By J. C. FORBES

It is generally agreed that the urinary excretion of iodine following the oral administration of a single dose of iodized fatty acids is considerably slower than after the ingestion of sodium or potassium iodide. Bröking (1) found on the average 75 per cent of the iodine of a single dose of potassium iodide in the urine during the next twelve hours. On the other hand, only 35–50 per cent of the iodine of Sajodin (calcium iodobehenate) was excreted in the urine by the eighty-fourth hour. Seven to 10 per cent of the latter preparation was excreted in the feces while only traces of the former were excreted by way of the bowel. Bonanni (2) found urinary iodine excretion to reach a maximum in about three hours following the ingestion of sodium or potassium iodide while it required seven to eight hours for maximum urinary excretion to be reached following the administration of iodized fatty acids. Bröking (1) showed that maximum excretion following the ingestion of iodized fatty acids might not be reached until the twelfth hour. Osborne (3) showed that the rate of iodine excretion in the urine was independent of urine volume, and reached a maximum in two to four hours following the administration of potassium iodide. McLean (4) found more iodine in the fatty tissues

* Received June 16, 1948, from the Department of Biochemistry, Medical College of Virginia, Richmond, Va.

of the body of rabbits following the administration of calcium iodobenhenate than after an equivalent amount of potassium iodide. Peabody (5) found no consistent difference in the iodine content of various body tissues of rabbits following the oral administration of potassium iodide and of ethyl diiodobrassidate over a considerable period of time. However, total iodine retention was a little greater in the case of the iodized fatty acid derivative.

Since there is no marked storage of iodized fatty acids in the body and since they are well absorbed from the gastrointestinal tract, repeated dosage with these iodized fatty acids should lead finally to approximately the same urinary iodide excretion per twenty-four hours as after an equivalent amount of sodium or potassium iodide. The present investigation was started to determine if this was the case and if the rate of excretion would be more constant in the case of the iodized fatty acids than in the case of the inorganic iodides.

EXPERIMENTAL

Five iodine preparations have been studied: potassium iodide, ethyl diiodobrassidate, calcium monoiodostearate, a calcium salt of mixed iodized fatty acids obtained by the fractional distillation of the fatty acids of hydrolyzed linseed oil, and stearolic acid diiodide. The preparation obtained from linseed oil undoubtedly was a mixture of mono-, di-, and triiodo fatty acids with possibly some saturated acids also. Its iodine content was only 33%. No free or inorganic iodine could be demonstrated in any of the organic preparations studied.

Urinary iodine was determined by the method of Gerard and Raunet (6). Preliminary experiments with the calcium salts of the iodized fatty acid showed that, for maximum absorption, they should be administered in a finely divided state. Consequently, in all the experiments reported, the organic iodine preparations were ground with corn starch and a weighed amount of the finely divided mixture put into hard gelatin capsules for oral administration. Although a number of subjects were used in studying some of the preparations, the results presented were all obtained on the same subject, the work extending over a period of a year. The results obtained with the stearolic acid diiodide are not included as they were very similar to those obtained with ethyl diiodobrassidate.

Figure 1 shows the urinary iodine excretion following the oral administration of a single dose of the various preparations. It will be seen that the iodine excretion reached a maximum earlier after potassium iodide than after any of the other preparations. The calcium salt of the mixed iodized fatty acids was next. In one subject, not shown in the charts, urinary iodide excretion following the ingestion of ethyl diiodobrassidate closely approximated that following the ingestion of the mixed iodized fatty acids, but this was usually not the case. The percentage of the total iodine intake excreted in the urine during the first twenty-four hours following the

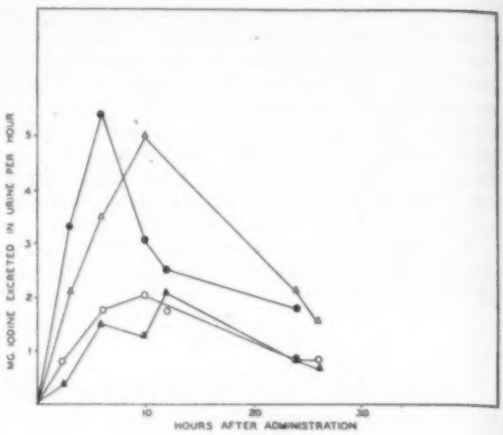

Figure 1.

●—After 88 mg. of iodine as KI. Average 2 exp.
△—After 86 mg. of iodine as Ca salt of mixed iodized acids. Average 2 exp.
○—After 86 mg. of iodine as Ca monoiodostearate. Average 2 exp.
▲—After 77 mg. of iodine as ethyl diiodobrassidate. Average 4 exp.

ingestion of a single dose of calcium monoiodostearate, ethyl diiodobrassidate, and stearolic acid diiodide was much less than in the case of potassium iodide or of the calcium salt of the mixed iodized fatty acids. However, when the various preparations were administered for several days the difference in total urinary iodine excretion per twenty-four hours became quite negligible (Figs. 2 and 3). It will be noted that after iodine administration for

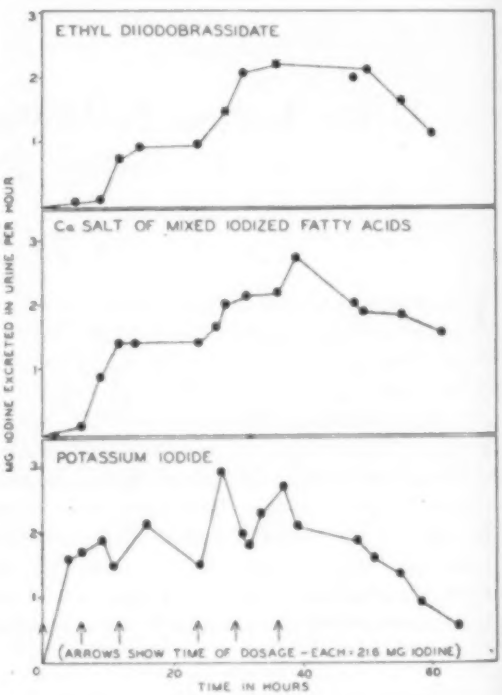
ETHYL DIIODOBRASSIDATE
Ca SALT OF MIXED IODIZED FATTY ACIDS
POTASSIUM IODIDE
MG IODINE EXCRETED IN URINE PER HOUR
(ARROWS SHOW TIME OF DOSAGE - EACH = 216 MG IODINE)
TIME IN HOURS

Figure 2.

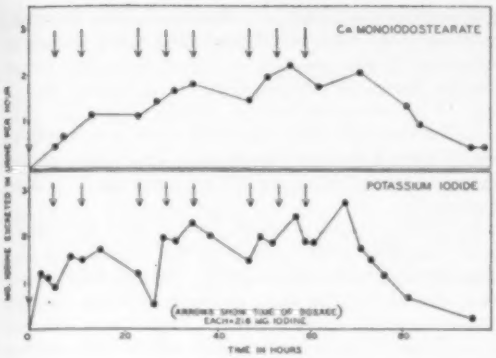

Figure 3.

a couple of days the urinary excretion rate became considerably more constant in the case of the organic iodine preparations than in the case of potassium iodide. As far as could be determined, all of the iodine in the urine was present as the iodide. Repeated attempts to show the presence of iodized fatty acids in the urine failed, even during the periods when urinary iodine elimination was at a maximum.

Since the rate of iodine excretion in the urine, in the case of a normal functioning kidney, must reflect the level of inorganic iodine in the plasma, it is safe to assume that the inorganic iodine level of the plasma obtained from the administration of repeated doses of iodized fatty acids would be more constant than from the administration of inorganic iodides. Such a result would also be expected from the fact that inorganic iodides are rapidly absorbed and eliminated, while, in the case of the iodized fatty acids, the liberation of inorganic iodine into the plasma is a function of the absorption and rate of metabolism of the iodized fatty acid. In view of this, it would appear that iodized fatty acids should offer definite therapeutic advantages over inorganic iodides in the treatment of such conditions as arteriosclerosis and hyperthyroidism. A recent article by Wolff and Chaikoff (7) is of considerable interest in this connection. These authors have shown that as long as the plasma inorganic iodine level is kept above 20–35 gamma per cent, organic binding of iodine by the thyroid gland is blocked, but as soon as the level drops below this critical level, formation of thyroxine and diiodotyrosine begins again. The more constant maintenance of this critical level without danger of excessive concentrations being reached at times should be considerably easier with the administration of iodized fatty acids than with inorganic iodides.

SUMMARY

The excretion of iodine in the urine following the oral administration of potassium iodide, ethyl diiodobrassidate, calcium monoiodostearate, stearolic acid diiodide, and the calcium salt of mixed iodized fatty acids prepared from the fatty acids of linseed oil has been studied.

Following the oral administration of a single dose, urinary iodine excretion was more rapid after potassium iodide than after the iodized fatty acids. However, when repeated doses of the various preparations were administered over several days, urinary excretion per twenty-four hours became roughly the same for all of the preparations studied.

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Artificial Inoculation of Rye with Ergot. A Susceptible Rye and the Control of Ergot Beetles*

By RALPH W. LEWIS

A strain of tetraploid Rosen rye was shown by artificial inoculation to be very susceptible to ergot. The ergot beetle, whose larvae eat the developing sclerotia, can be largely controlled by the use of DDT dust.

IN A previous paper (1) and in a patent (2) the author has described a method for the artificial inoculation of rye with ergot. The novelty of the method consists of spraying the blooming rye plants with a heavy sugar-spore-suspension rather than a plain water suspension of spores. The spores in droplets of a sugar suspension remain viable and capable of causing infection for several days after being sprayed on the rye spikes. Spores in a plain water suspension lose their ability to germinate in a short time and thus

* Received May 18, 1948, from the Department of Biological Science, Michigan State College, East Lansing, Mich.

can only cause infection if they fall on a stigma when first sprayed on the rye.

The possibility of making this method commercially feasible depends upon the determination of the optima of several variables as noted previously (1). One of the variables is the variety of rye employed. This paper describes a strain of rye which, because of its susceptibility and habit of growth, appears to be more desirable than Common Rosen rye (*Secale cereale*) for the production of ergot. Results on the use of DDT to control the ergot beetle, whose larvae do considerable damage to the developing sclerotia, are also reported. Due to various circumstances this research was terminated before all the planned experiments were carried out, consequently the results are fragmentary and all conclusions tentative.

EXPERIMENTAL

In 1944 a number of varieties of rye were tested for a susceptibility to ergot by spraying them with a sugar-spore-suspension. One variety called Double Rosen, a tetraploid, was much more susceptible than the others. It had 48% of the heads infected with at least one sclerotium while the sprayed Common Rosen, a diploid, had 28% and the unsprayed Common Rosen had less than 1%. These trials consisted of 15-ft. rows of each variety repeated four times. They were sprayed only once.

The Double Rosen rye was secured from Dr. H. H. Love, Department of Agronomy, Cornell University. It was different from Common Rosen rye in that many of the florets did not produce seed. Because the spike was light due to the failure of many seeds to ripen and because the straw was stiff, the spike remained upright even after the seeds and sclerotia had matured. The uprightness of the spike would be a desirable trait if a mechanical harvester were to be used to gather the sclerotia direct from the field. The partial sterility of this strain of Double Rosen rye makes available a larger amount of food for the developing sclerotia and it appears to cause the flowers to remain open longer, thus exposing them to infection for a longer period of time. The stigmas of the Double Rosen were longer than those of the Common Rosen rye. The average length of stigma (five of each measured) was 5.8 mm. for the former and 4.3 mm. for the latter. Considering that the spores germinate on the stigma to bring about infection this extra length is important. More of the stigma is exposed at the time of blooming and much more remains exposed to the outside by protruding between the palea and lemma after the flower closes.

A second lot of the Double Rosen seed was requested from Dr. Love for the 1945 season. A large planting was made from this new seed. The seed proved to be different from that used the previous year. The plants appeared identical with Common Rosen, that is, the spikes all drooped when ripe and the stems were not so robust. All comparative results between Common and Double Rosen for this season were destroyed by burning of the plants with arsenate dust or by wind. Observations in the field, however, led to the conclusion that there was little difference in the amount of infection in this lot of Double Rosen as compared to the Common Rosen rye. The previous year's results were so pronounced that the difference was obvious in the field before the plants were harvested, thus large differences in yield of sclerotia can be recognized by field observation.

In order to use the strain of rye that gave such good results in 1944, seed from the original lot was planted for inoculation in 1946. Small plots 6 rows wide and 15 ft. long were laid out adjacent to each other along the same 6 rows. Plot 1 was sprayed three times in the same morning with a high quality (50% germination) spore suspension diluted 1–15 as described in a previous paper (1). Plot 2 was unsprayed and Plot 3 was sprayed twice with the same spore suspension in the late afternoon five days after Plot 1. Both plots were sprayed during the seven- to nine-day blooming period in early June. As soon as the "honey-dew" began to be evident, which was about fifteen days after inoculation, ergot beetles (*Acylomus ergoti* Casey) were present by the hundreds. The north three rows of each plot were dusted once with 5% DDT.

RESULTS AND DISCUSSION

Results of three plots selected as typical are presented in Table I. It will be noted that the control plot has a high percentage of ergot infection as compared to the control plots reported previously (1). This can be expected to occur when dealing with small plots of a very susceptible rye. The longer blooming period of this rye, the larger number of insects that transmit the spores, and the blowing of the spore suspension probably account for this large percentage of infection in the control plots. There is, however, 20 to 40 per cent more infection on the treated plots. The highest infection of Common Rosen rye obtained in previous work (1) was 60 per cent in the treated plots with 10 per cent in the untreated plots. This high percentage of infection on the Common Rosen rye was secured by spraying the plants on five different days with a spore suspension approximately 15 times as concen-

TABLE I.—THE EFFECT OF DDT UPON THE ARTIFICIAL INOCULATION OF DOUBLE ROSEN RYE WITH ERGOT

Plot	No. Times Sprayed	Insecticide	Per Cent Spikes Infected with Ergot
1*a*	3	DDT	88
1*b*	3	None	89
2*a*	Control	DDT	50
2*b*	Control	None	47
3*a*	2	DDT	73
3*b*	2	None	72

trated as used in the work described in the present paper. The Double Rosen was more highly infected despite fewer applications of spores in a much greater dilution.

The DDT had no effect on the number of sclerotia which developed in each plot, but it did have a pronounced effect upon the number of insects as measured by their effect on the sclerotia. The samples from 1*a*, 2*a*, and 3*a* were thrown together as were 1*b*, 2*b*, and 3*b*. The percentage of sclerotia with insect burrows was 68 for the undusted plots and 14 for the dusted plots. This difference is paralleled by a field count of 28 adult beetles on two undusted rows while there were only 2 beetles on comparable dusted rows.

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Influence of *p*H on the Diastatic Activity of Fungal and Malt Amylases*,†

By R. S. J. RAU and A. SREENIVASAN

The effects of changes in *p*H on the diastatic activity of fungal and malt amylase preparations are reported and the bearing of the results on their use as pharmaceuticals is discussed.

A NUMBER of workers (1) have investigated the action of amylases at various hydrogen-ion concentrations for studying the optimum *p*H ranges over which they are active and, while it is generally recognized that amylases are inactive at high alkaline and particularly acidic *p*H ranges, no work has been reported on the effects of a sequence of *p*H changes similar to that existing in the gastric and duodenal regions. Such a study is of significance especially with fungal and malt amylases which have been the commonest among the diastatic preparations used in medicine (2). Takamine (3) has referred to the relatively greater stability and resistance to acid of the amyloclastic enzyme of taka-diastase as compared to that of malt. Ohlsson and Swaetichin (4) have observed loss in power of taka-diastase to hydrolyze starch in acid ($< p$H 2.0) or alkaline ($> p$H 11.5) solutions and suggest that reactivation or regeneration of the enzyme may take place on restoration of the *p*H to the optimal value by neutralization. The mechanism of inactivation of taka-diastase has also been studied by Yamashiki (5) and, more recently, by Akabori, *et al.* (6); the latter authors report that the amylase is inactivated in thirty minutes at *p*H 2.0, that it is reactivated by adjustment to *p*H 5.0 and that, therefore, activation and reactivation are primarily reversible processes.

In order to investigate this property further under conditions more or less similar to those *in vivo* and also to provide data which might serve as a basis for the therapeutic use of fungal and malt amylase preparations, the following studies were undertaken.

EXPERIMENTAL

Materials and Methods.—The amylase preparations employed in these studies were both prepared in the laboratory: for the *fungal amylase*, a strain of *Aspergillus oryzae*, No. 558 from the National Collection of Type Cultures, Bangalore, India, was inoculated on a previously cooked and cooled bran medium and growth allowed to proceed in shallow trays at 30° for two and a half days. The moldy bran was then extracted with ten volumes of 2.5% saline and the enzyme precipitated from the filtered extract by addition of alcohol to a concentration of 70%. *Malt diastase* was obtained by extraction with water of three-day germinated barley at room temperature (28°) followed by precipitation of the enzyme from solution with 70% alcohol. The fungal and malt enzyme preparations obtained as above were purified by dissolution in water followed by reprecipitation from 70% alcohol; the dry products had activities, respectively, of 16,300 and 13,500 Lintner units per gram (7).

Saccharogenic activities of the enzyme preparations, under specified conditions, were measured by the iodometric determination of reducing sugars (8) formed after half-hour hydrolysis at 37.5° acting on 2% maize starch solution. Suitable controls were always run and the reducing values recorded are those obtained after allowance for blank readings.

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† Acknowledgment is made to the Provincial Industrial Research Committee of the Bombay Government Department of Industries for a grant in support of this work.

Influence of *p*H on Amylase Activity.—The saccharifying power of the enzyme preparations was studied at *p*H 1.8, corresponding to the concentration of gastric hydrochloric acid, at *p*H 3.4 at which *p*H *alpha*-amylase is known to be inactivated (9, 10), at *p*H 4.8, the optimum *p*H for both the enzymes, and, finally, at *p*H 8.0, corresponding to the *p*H of the intestinal secretions. The buffer solutions employed were Clark's *M*/5 potassium chloride-hydrochloric acid (1.8), Clark's *M*/5 potassium hydrogen phthalate-hydrochloric acid (3.4), Walpole's *N*/1 sodium acetate-acetic acid (4.8), and Sorensen's *M*/15 phosphate (8.0). The reaction mixture consisted of 10 ml. of 4.0% starch solution, 2 ml. of the appropriate buffer solution, 3 ml. of water, and 5 ml. of enzyme solution containing 2 mg./ml. of the dry preparation. The results (Table I) are expressed in terms of milligrams of maltose hydrate per 100 ml. of the starch-enzyme mixture.

TABLE I.—ACTIVITIES OF FUNGAL AND MALT AMYLASES AT VARIOUS *p*H

	Mg. Maltose Hydrate Formed per 100 Ml. of Reaction Mixture After 1/2 Hr. Digestion			
	*p*H 1.8	*p*H 3.4	*p*H 4.8	*p*H 8.0
Fungal amylase	68	136	408	102
Malt amylase	0	85	323	119

It may be seen that there is pronounced loss in enzyme activity on either side of the optimum *p*H; the decrease in activity is more in acid ranges and, with malt amylase, there is complete inactivation at *p*H 1.8.

Rate of Inactivation at *p*H 1.8.—The decrease in activity in presence of acid (Table I) may be due to either adverse effect of *p*H or to inactivation of the amylases. To ascertain this further, a study was made of the rate of loss in enzymic activity at *p*H 1.8. Eighteen milliliters of the enzyme solution, containing 5 mg. of dry preparation per ml., were diluted with 24 ml. of water and treated with 3 ml. of buffer (*p*H 1.8). At stated intervals, 5-ml. aliquots of the reaction mixture, corresponding to 10 mg. of the enzyme preparation, were pipetted into a mixture of 10 ml. of 4% starch solution and 5 ml. of buffer (*p*H 4.8). The saccharogenic activity was measured as before. The results for fungal amylase are given in Table II.

It is interesting to note that, although fungal amylase loses a considerable percentage of its activity at *p*H 1.8 for half an hour (Table I), the activity returns to almost the normal value on restoration of the *p*H to the optimum for the enzyme. There is, however, slow and stepwise degradation of the enzyme on continued contact at low *p*H but there is little doubt that inactivation at acid *p*H ranges is largely of a reversible type.

When these experiments were repeated with malt enzyme, the striking observations were made that malt amylase was almost instantaneously inactivated at *p*H 1.8 and that this inactivation was irreversible, there being no activity even after restoration to optimum *p*H. It was therefore obvious that malt amylase was irreversibly destroyed at highly acidic *p*H.

Action of Pepsin.—Enzymes being in the nature of proteins, the activities *in vivo* of the amylase preparations may, it was felt, be adversely affected by the pepsin of the stomach. In the following experiments, peptic digestion of 5-ml. lots of mold and malt amylase solutions containing 5 mg. of enzyme per ml. was carried out at *p*H 1.8 and at 37.5° by addition of 1 ml. of buffer (1.8) and 5 ml. of a solution of pepsin, containing 6 mg./ml. of a commercial preparation (B. D. H.) and after dilution to 15 ml. At the end of half an hour, saccharogenic activity was determined as usual by pipetting out 5 ml. of the reaction mixture, corresponding to 10 mg. of the amylase, into a mixture of 10 ml. of 4% starch solution and 5 ml. of buffer (*p*H 4.8). Dialysis of the peptic digests was tried before adjustment to *p*H 4.8 but the alternative procedure detailed here was found to be easier and more satisfactory, the desired *p*H change being effected by suitable adjustment of the quantities of the buffers as indicated above. The results together with those of control experiments at *p*H 1.8 without pepsin and at optimum *p*H are presented in Table III.

TABLE II.—RATE OF INACTIVATION OF FUNGAL AMYLASE AT *p*H 1.8

Time of contact of the enzyme with the buffer (*p*H 1.8) in minutes	0	2 1/2	5	10	20	30
Milligrams maltose hydrate formed per 100 ml. of reaction mixture at end of 1/2 hr., *p*H 4.8, 37.5°	391	374	374	357	357	340

TABLE III.—ACTION OF PEPSIN ON AMYLASES

	Mg. of Maltose Hydrate Formed per 100 Ml. of Reaction Mixture After 1/2 Hr. Digestion	
	Fungal Amylase	Malt Amylase
Amylase + pepsin (*p*H 1.8 for 1/2 hour followed by *p*H 4.8)	357	51
Amylase only (*p*H 1.8 for 1/2 hour followed by *p*H 4.8)	357	0
Amylase only at *p*H 4.8	357	316

The results show that pepsin does not affect amylase activity. In fact, malt amylase is slightly protected by pepsin against inactivation by acid, retaining thereby about one-sixth of its original activity.

Action of Pancreatin.—As fungal amylase was regenerated after inactivation by acid, the activity of the amylase was studied under conditions similar to those prevailing in the small intestine. The combined action of fungal and pancreatic amylases was also studied at *p*H 8.0 (Table IV). The reaction mixture consisted of 10 ml. of 4% starch solution, 5 ml. of buffer (*p*H 8.0), and 5 ml. of enzyme solution containing 2 mg./ml. of fungal amylase or malt amylase with and without pancreatin (Stearn's) in similar amount.

TABLE IV.—ACTION OF PANCREATIN

	Mg. of Maltose Hydrate Formed per 100 Ml. of the Reaction Mixture After ½ Hr. Digestion
Fungal amylase	102
Pancreatin	34
Fungal amylase + pancreatin	187
Malt amylase	119
Malt amylase + pancreatin	204

As in the case of pepsin, there is evidently a protective effect exerted by pancreatin on the diastassis of starch by fungal or malt amylase at *p*H 8.0. The possible mutual synergism between these amylases also should not be overlooked.

That there could be pronounced synergism between amylases from different sources was shown in other studies, to be published elsewhere, using highly purified laboratory preparations of amylases of vegetable, animal, bacterial, and fungal origin, either singly or in combination. A summarized statement of the results obtained in a typical set of experiments is given in Table V. The reaction mixture consisted of 50 ml. of 4% starch solution, 10 ml. of appropriate buffer, and 10 ml. of enzyme solution containing 10 mg. of the enzyme product or mixture of enzyme products per ml., finally made up to 100 ml. with water. Hydrolysis was carried out either at the optimum *p*H in the case of individual enzymes or at a suitable intermediate *p*H for mixtures; this had to be done since pancreatic amylase had very low activity at *p*H 4.8. Maximum saccharification was reached in seventy-two to one hundred and forty-four hours in all cases. The limit of saccharification recorded in Table V represents the maximum of the reducing sugars formed expressed as per cent on the value obtained by hydrolysis of starch solution with concentrated hydrochloric acid for one hour at 15 lbs. steam pressure (125°); this latter treatment is known to result in complete breakdown of starch and dextrins to reducing sugars (11).

TABLE V.—SACCHARIFICATION OF STARCH BY AMYLASES

Enzyme or Enzymes	*p*H of Hydrolysis	Period of Hydrolysis, Hr.	Limit of Maltose Formation, %
Fungal amylase	4.8	96	57.8
Malt amylase	4.8	72	69.3
Pancreatic amylase	7.0	144	47.0
Fungal amylase + malt amylase (9:1)	4.8	72	65.3
Fungal amylase + pancreatic amylase (1:9)	5.8	120	67.9*
Malt amylase + pancreatic amylase (1:9)	5.8	72	68.8

DISCUSSION

Inactivation of malt amylase by acid, especially in concentration similar to that of gastric hydrochloric acid immediately after food ingestion, is almost instantaneous and is, besides, irreversible. It would therefore follow that, except in so far as they provide predigested carbohydrate and protein foods, preparations of malt have little value as diastatic agents in medicine and that there is no practical significance in the determination of diastatic value of malt extracts as laid down in official methods (12). The value of malt diastase, when employed with enteric coatings that disintegrate in the duodenum (13), is also perhaps limited because of low activity in the *p*H range of the duodenum.

With fungal amylase, on the other hand, though there is a certain amount of slow and stepwise inactivation at low acidic *p*H, the enzyme is quickly regenerated on restoration of *p*H to its optimum. Even at *p*H 8.0, there is improved activity due to the synergistic effect of pancreatic amylase.

The change in reaction from acidic to alkaline side, during the period the food material passes from the gastric to the duodenal region, may be expected to be gradual, passing through the optimal range for the fungal amylase; hence, it may be expected that the cumulative action of the enzyme will be considerably more than *in vitro* digestibility trials, such as those reported here, would indicate.

SUMMARY

1. Diastatic activities of fungal and malt amylases are affected adversely on either side of the optimum *p*H 4.8. Malt amylase loses its diastatic power at *p*H 1.8.

2. Malt amylase is irreversibly inactivated almost instantaneously at *p*H 1.8, whereas with fungal amylase at low acid *p*H ranges inactivation is slow and stepwise besides being reversible. Thus, the enzyme could be regenerated by restoration of *p*H to optimum.

3. Pepsin has no inactivating influence on the amylases. On the other hand, some protection against inactivation is noticeable.

4. Similar protection against inactivation at alkaline *p*H ranges is afforded by pancreatin. Marked synergism is also observed in the saccharification of starch by fungal or malt amylases in combination with pancreatic amylase.

5. The significance of the results obtained is discussed in relation to the use of amylase preparations in medicine.

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Action of Dolophine on the Animal Uterus*

By CLARENCE E. POWELL and K. K. CHEN†

Results of a study of the action of Dolophine on the isolated uteri of certain laboratory animals are reported. The action of Dolophine appears to depend on the initial uterine activity. It inhibits those which are in rhythmic movements, but stimulates others which are stationary.

DOLOPHINE (Methadon, Lilly) is a new analgesic of German origin, disclosed after V-J Day by the Technical Industrial Intelligence Committee, U. S. Department of State (1). Its resemblance to morphine and its addiction liabilities were elucidated by Scott and Chen (2), and Isbell, Wikler, Eddy, Wilson, and Moran (3), respectively. The spasmolytic action of Dolophine on the rabbits' and rats' uteri was investigated by Kirchhof, Uchiyama, and David (4, 5).

Although morphine is frequently used in labor, its action on the puerperal uterus is not completely understood. Krueger, Eddy, and Sumwalt (6), who have summarized the information on opium alkaloids, believed that any effects of morphine on the human uterus in labor were mainly the result of the central action. In animals, morphine increases the tone of excised and intact uteri in low concentrations, but decreases the tone in high concentrations (6).

Dolophine has been applied in obstetrics, but was found to cause depression of fetal respiration (7, 8). Notwithstanding, a thorough study of its action on the uteri of different species of animals would be desirable. This prompted the presentation of the present data on the isolated uterus, the uterus *in situ*, and the uterus in spasm induced by stimulating substances. A 0.5–1 per cent solution of Dolophine, freshly prepared, was employed throughout the experiments.

Isolated Uterus.—Uteri of nonpregnant rabbits, rats, golden hamsters, cats, and guinea pigs were removed, and short strips suspended in aerated Ringer's or Tyrode's solution. Their activities were recorded on a smoked drum. After a control period of observation, Dolophine was added to the bath, and the response watched until recovery took place. The rabbits used in this study were pretreated with estrone, because, as shown previously by Wick and Powell (9), this made them more reactive to different drugs.

The results are summarized in Table I. It should be noticed that of 19 strips of rabbits' uteri, 2 showed rhythmic movements while 17 were inactive. Dolophine in various concentrations stimulated the nonrhythmic uteri, but inhibited the uteri in rhyth-

TABLE I.—ACTION OF DOLOPHINE ON ISOLATED UTERUS AND UTERUS *in situ*

	Animal	Condition of Uterus	Dose Range	Number of Observations	Results
Isolated uterus	Rabbit	Nonrhythmic	1:650,000–1:10,000	17	Stimulation
		Rhythmic	1:50,000–1:10,000	2	Inhibition
	Guinea pig	Nonrhythmic	1:1,000,000–1:10,000	7	No action
			1:1,000,000–1:10,000	6	Stimulation
		Rhythmic	1:50,000–1:10,000	2	Inhibition
	Rat	Rhythmic	1:400,000–1:10,000	9	Inhibition
	Hamster	Rhythmic	1:100,000–1:5,000	6	Inhibition
			1:200,000	2	No action
	Cat	Rhythmic	1:200,000–1:10,000	4	Inhibition
Isolated uterus in spasm	Rabbit	Treated with mecholyl	1:100,000–1:7,500	10	Inhibition
		Treated with ergonovine	1:40,000–1:5,000	11	Inhibition
	Guinea pig	Treated with pituitary extract	1:100,000–1:50,000	4	Inhibition
			1:50,000–1:25,000	4	Slight inhibition
Uterus *in situ*	Rabbit	Nonpregnant, rhythmic	0.5–2 mg./Kg.	6	Inhibition
			0.5 mg./Kg.	1	Stimulation
			0.25 mg./Kg.	1	No action
	Cat	Nonpregnant, feebly rhythmic	1.0 mg./Kg.	1	Stimulation
			1.0 mg./Kg.	1	No action
			0.5 mg./Kg.	1	No action
	Dog	*Post partum*, rhythmic	5.0 mg./Kg.	1	Stimulation
			5.0 mg./Kg.	1	Slight inhibition
			3.0 mg./Kg.	1	No action
			2.0 mg./Kg.	1	Slight stimulation

* Received May 27, 1948, from the Lilly Research Laboratories, Indianapolis, Ind.

† We are indebted to Mr. Lester Le Compte, Jr., for his valuable assistance in these experiments.

mic activity. The duration of stimulation lasted four to five minutes and that of inhibition, thirteen to fifteen minutes. Examples of such opposite responses are shown in Fig. 1.

Fig. 1.—Action of Dolophine on isolated rabbits' uteri. Note the stimulation on an inactive strip, and the inhibition of the rhythmic strip.

With guinea pigs, 13 out of 15 strips were inactive at the start, 6 of which reacted to Dolophine by contraction, while 7 remained inactive. The other 2 strips in rhythmic activity relaxed upon the addition of Dolophine. The rat uteri were all in rhythmic activity, and all showed inhibition to Dolophine. All 8 strips of the hamster uteri were in rhythmic activity; 6 of them relaxed and the remaining 2 failed to respond. The inhibitory action of Dolophine in rats and hamsters is well illustrated in Fig. 2. Inhibition was also the rule with the 4 strips of the cat uteri.

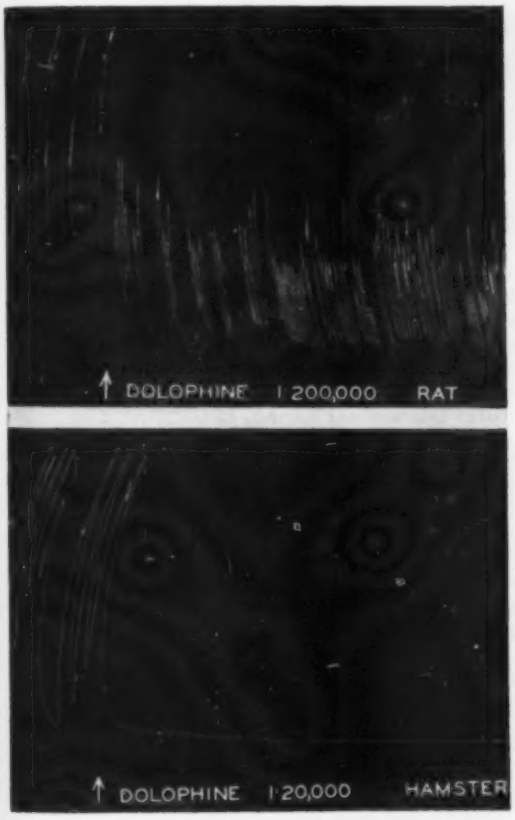

Fig. 2.—Inhibitory action of Dolophine on the isolated rat's and hamster's uteri.

Uterus in situ.—Uterine horns of 8 rabbits and 3 cats were each connected to a writing lever with a string through 2 pulleys—for kymographic recording. The uterine movements of 4 dogs, one to three days *post partum*, were each registered through a balloon inserted into the uterine cavity, and attached to a tambour. All animals were anesthetized with ether. Dolophine in various doses was injected intravenously.

As shown in Table I, 6 of 8 nonpregnant rabbits responded to Dolophine by inhibition—the dose being between 0.5–2 mg. per Kg. An example is given in Fig. 3. One animal showed stimulation of uterine movements with a dose of 0.5 mg. per Kg. The eighth rabbit was without any effect with a dose of 0.25 mg. per Kg.

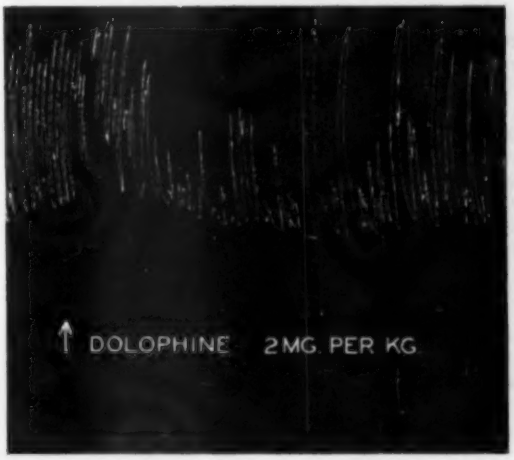

Fig. 3.—Inhibitory action of Dolophine on the rabbit's uterus *in situ*.

Less certain was the action of Dolophine on the cat's uterus *in situ*. One was stimulated with a dose of 1 mg. per Kg., but another failed to respond with the same dose. A dose of 0.5 mg. per Kg. was also without effect on a third animal. It must be mentioned that the initial uterine activity of these animals was feeble.

Similar uncertainty of results was noted on the uterus of *post partum* dogs. Dolophine by slow intravenous injection in the dose of 5 mg. per Kg. caused stimulation in 1 animal, but slight inhibition in another. Small doses were either ineffective or slightly stimulating.

Isolated Uterus in Spasm.—Spasm was induced in isolated rabbits' and guinea pigs' uteri by mecholyl iodide, ergonovine maleate, or pituitary extract. It is obvious from Table I that inhibition uniformly occurred with the isolated rabbit's uterus when Dolophine was added at the peak of action of mecholyl or ergonovine. With the isolated guinea pig's uterus, Dolophine in concentrations of 1 : 100,000–1 : 50,000 released the spasm caused by pituitary extract, but in concentrations of 1 : 50,000–1 : 25,000 it exerted a slight stimulating action, probably indicating a synergistic action with pituitary extract.

DISCUSSION

The action of Dolophine on the uterus appears to depend on the state of its initial activity. If it is in rhythmic movements, the drug causes relaxation; but if it is inactive at the start, the drug usually produces contractions. These opposite effects occur uniformly with the isolated uteri of rabbits, guinea pigs, rats, hamsters, and cats.

As to the uterus *in situ* in etherized rabbits, which shows rhythmic activity, Dolophine is inhibitory in the majority of experiments. The action of the drug on the nonpregnant cat's uterus or the dog's *post partum* uterus is less certain; stimulation of a mild degree seems to be more frequent than slight inhibition. In all the anesthetized animals, the central action may contribute to the modification of the uterine response.

The action of Dolophine is probably not on the autonomic nervous system of the uterus. The lack of unidirectional response would make such a possibility unlikely. It seems that the drug is endowed with a direct muscular action capable of relaxing an active, but stimulating an inactive, uterus. This may also explain the release of spasm by Dolophine induced by mecholyl iodide, ergonovine maleate, or pituitary extract. Only in relatively strong solutions does Dolophine show a slight synergistic action with pituitary extract on the isolated guinea pig's uterus.

SUMMARY

1. The action of Dolophine on the isolated uteri of rabbits, guinea pigs, rats, golden hamsters, and cats appears to depend on the initial uterine activity. It inhibits those which are in rhythmic movements, but stimulates others which are stationary.

2. Dolophine inhibits rabbits' rhythmic uteri *in situ* in the majority of experiments. The results are less certain with cats' uteri which are feebly rhythmic, and dog's *post partum* uteri which are definitely rhythmic.

3. Dolophine releases the spasm of isolated rabbits' uteri, induced by mecholyl or ergonovine. Weak solutions of Dolophine cause relaxation of guinea pigs' uteri in spasm induced by pituitary extract, but relatively stronger solutions may result in a slight stimulation.

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1949 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1949 Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to the Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, Dr. Robert P. Fischelis, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1949 Award, nominations must be received before January 1.

Establishment of the iodine award was first announced at the Milwaukee convention of the AMERICAN PHARMACEUTICAL ASSOCIATION which administers the competition.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During a period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council and which functions under prescribed rules.

Should the award committee fail to find a suitable recipient in any year, two awards may be made during the next year if suitable recipients are selected.

Microcrystallographic Differentiation of *dl*-Amphetamine Sulfate (Benzedrine) and *d*-Amphetamine Sulfate (Dexedrine)*

By GEORGE L. KEENAN†,‡

A microscopic method for quickly and accurately differentiating these two forms of amphetamine sulfate has been devised, applying the immersion method and microchemical test with a suitable reagent. The *d*- and *dl*-forms were found to be markedly different in respect to their optical crystallographic properties as determined by the polarizing microscope. Crystalline addition products formed with gold chloride reagent showed that the microchemical test was equally as valuable for a rapid and accurate diagnosis.

THERE appears to be no record of a microscopic study of the *dl*- and *d*-forms of amphetamine sulfate. More recently Haley (2) described a chemical differentiation of several sympathomimetic amines, *dl*- and *d*-amphetamine sulfate being included among them. The occasion arose for a rapid and accurate method for distinguishing these two forms microscopically, applying both optical and strictly microchemical techniques. Both substances lend themselves to a study of this nature, the results being so striking as to justify calling them to the attention of the analyst.

***dl*-Amphetamine Sulfate (Benzedrine).**—In ordinary light, the microscopic examination shows that the substance is definitely crystalline, consisting largely of small, irregular particles, frequently elongated but lacking in any significant geometric forms. In this respect, this form is not as satisfactory for microscopic study as the *d*-compound. Crossed nicols revealed nothing significant and the fragments were too small to reveal interference figures by the conoscopic method. From the standpoint of the refractive indices, however, one value, the minimum index (α), is so frequently found lengthwise (oriented parallel to the vibration plane of the lower nicol) as to be very valuable in diagnosis. This index, $\alpha = 1.520$, is the only one measurable, the unsatisfactory cleavage precluding additional determinations that would be trustworthy.

Attention has already been called to the crystalline addition product formed with gold chloride reagent (1). The very thin, yellow, quadratic plates are quite striking when examined microscopically in ordinary light, frequently so thin as to exhibit faint interference colors. Many of the plates extinguish sharply when examined with crossed nicols (parallel polarized light) and revolution of the microscope stage, others remaining bright under the same conditions. The latter show a distinct biaxial interference figure by the conoscopic examination, two isogyres of the figure appearing in the field of view. The axial angle is small with negative optic sign.

***d*-Amphetamine Sulfate (Dexedrine).**—Unlike the racemic compound, this form, when examined microscopically in ordinary light, exhibits a platelike habit, many of the plates being 6–8-sided in outline, some elongated, usually lying on the broad face, occasionally tipped on edge and simulating the appearance of needles. With crossed nicols, many of the plates do not extinguish sharply when the microscope stage is revolved. The sign of elongation is negative. With the conoscopic examination, biaxial interference figures showing the axial plane frequently are observed, 2E being small and the optic sign positive. The refractive indices (applying the immersion method) are: $\alpha = 1.505$, $\beta = 1.545$, $\gamma = 1.608$, all ± 0.002. α and β usually found on plates lying on the broad face and α and γ on plates tipped on edge. The α and β values are most conveniently found and diagnostic for the substance.

Gold chloride reagent (5%) also forms a crystalline addition product with the *d*-compound but of a significantly different habit than that characteristic of the racemic form. Both compounds are readily soluble in the reagent, but in the case of the *d*-form, the crystalline addition product forms almost immediately, crystallizing out in the form of short, stout, prismatic rods and needles (parallel extinction with crossed nicols), and not quadratic plates as in the case of the *dl*-form. Furthermore, the plates of the racemic compound come out much more slowly, it frequently being necessary to add an additional amount to the reagent drop and scratch the slide with the rod. The rate of formation of the respective addition products with gold chloride is quite as significant as the marked difference in their habit.

SUMMARY

The *d*- and *dl*-forms of amphetamine sulfate exhibit marked differences microscopically, in respect to both the optical crystallographic properties and the difference in the habit and rate of formation of the crystalline addition products with gold chloride reagent. By these two

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Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
† The author wishes to acknowledge the valuable assistance of Dr. Thomas J. Haley, Los Angeles, Calif.; Smith, Kline & French Laboratories, Philadelphia; and Dr. Justin L. Powers, Editor of THIS JOURNAL. Acknowledgment is also due the Chemistry Department, Baldwin-Wallace College, Berea, Ohio, for use of apparatus.
‡ Present address: 165 Westwood Drive, Strongsville, Ohio.

TABLE I.—MICROSCOPIC DIFFERENCES OF *d*- AND *dl*-AMPHETAMINE

	Habit	Refractive indices α	β	γ
dl-Amphetamine SO_4	Irregular fragments	1.520	...	...
Gold salt	Quadratic plates			
d-Amphetamine SO_4	Plates, rods	1.505	1.545	1.608
Gold salt	Rods			

methods both compounds can be quickly and accurately differentiated.

Table I summarizes the significant microscopic differences.

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A Study of Phosphorus Uptake in the Rat Tooth by Radioactive Tracer Techniques*

By CATHERINE J. HURLEY and JOHN E. CHRISTIAN†

The results of a study to determine the rate of uptake of phosphate in rats' teeth following the intraperitoneal injection of potassium biphosphate containing radioactive phosphorus are reported. The techniques employed are described and the results obtained are discussed and tabulated.

A STUDY of phosphorus uptake in the rat tooth by radioactive tracer technique was considered necessary in view of subsequent experiments which were to be carried out using rat teeth which contained radioactive phosphorus. Such experiments required that the rat teeth be made sufficiently radioactive with a minimum amount of radioactive phosphate solution and in as short a time as possible.

A complete survey of the literature concerning phosphorus uptake in teeth using radioactive tracers indicates that no data have been reported on the rate of phosphorus uptake when the radioactive phosphate solution is injected intraperitoneally. The intraperitonael injection of radioactive materials is considered by the authors to be the most efficient method available for this type of work since this method is rapid, complete dosage is assured, and rapid absorption results. In addition, the possibility of radioactive contamination is less by this method.

LITERATURE REVIEW

Greenberg (1) described the work of Chievitz and Hevesy, who added phosphorus to food and demonstrated that an appreciable part found its way to the bones, teeth, muscle, and other organs.

Hevesy, Holst, and Krogh (2) found that in the rat the labeled phosphorus was deposited in close proximity to the pulp from which it is derived and that it was equally distributed throughout the incisors.

Hevesy (3) developed a technique to avoid corrections for the difference in the absorption of the beta rays emitted by samples of different thickness and composition.

Hevesy, Levi, and Rebbe (4) injected radioactive phosphorus subcutaneously into rabbits. In fifty days, the incisor dentin phosphorus of the apical and medial parts was almost completely replaced.

Hevesy and Armstrong (5) injected radioactive phosphate subcutaneously into cats, removed the teeth, and separated the enamel. They found there was a slow exchange of phosphorus but no ability of the enamel to undergo significant changes after eruption as a result of nutritive alterations.

Armstrong (6) agitated tooth samples in radioactive phosphate solutions to determine the rate of exchange of phosphate.

Armstrong and Barnum (7) separated the dentin from the enamel of cats that had been injected subcutaneously with radioactive sodium phosphate. They found that the enamel exchanged phosphate at a rate approximately 5% that of dentin.

Armstrong (8) also calculated the relative uptakes of phosphorus and calcium in the incisor dentin and enamel and the molar dentin and enamel.

LeFevre and Bale (9) measured the amount of marked phosphorus deposited in the teeth of rats killed four to twenty hours after having been fed radioactive phosphorus.

Manly and Bale (10) also determined the phosphorus turnover in the inorganic portion of rat teeth.

Manly, Hodge, and Van Voorhis (11) cut grooves in the teeth of a dog which had been given radioactive phosphorus by stomach tube. There was no difference in the phosphorus content of the scarred and unscarred teeth.

* Received March 3, 1948, from the Research Laboratories, Purdue University, School of Pharmacy, Lafayette, Ind.
† We are indebted to Mr. D. J. Tendam of the Physics Department of Purdue University for assistance with the measuring instruments used in this research.

Johannson, Falkenheim, and Hodge (12) studied the relative abilities of powdered dentin and enamel to adsorb ions from aqueous solutions. It was shown that for short periods the dentin adsorbed more than the enamel.

In a further study, Hodge and Falkenheim (13) found that for longer periods there was a complete reversal of the order of adsorption: the enamel adsorbed more than the dentin.

Hodge, *et al.* (14), determined the amount of phosphate adsorbed by calcified tissues from the KOH-ethylene glycol solution used to extract the organic portion of teeth.

Sognnaes and Volker (15) and (16) worked with cats, dogs, and a monkey. They separated the enamel and the dentin of the teeth and determined the concentration of phosphate. The concentration in the dentin was inversely proportional to the density while the opposite seemed to hold for the enamel.

Sognnaes and Volker (17) also studied the amount of phosphorus in the maxillary and mandibular bones, as well as the teeth, of a cat that had been given radioactive phosphate by stomach tube.

Bevelander and Amler (18) used human teeth that had been sectioned on an abrasive wheel. They determined the activity in the different regions of the dentin.

Pedersen and Smidt-Nielsen (19) studied to what degree surface enamel took up phosphorus through the pulp and dentin.

Using X-ray film, McCauley and Gilda (20) determined the absorption of phosphate by the pulpless teeth of dogs. There was a striking decrease in the amount of labeled phosphorus in pulpless teeth.

The foregoing is a complete review of the literature that concerns work on the teeth using radioactive phosphorus.

EXPERIMENTAL

Since no data are available in the literature concerning the rate of uptake of phosphorus in rats' teeth on intraperitoneal injection of radioactive phosphate and since the amounts of active phosphate necessary to confer sufficient activity to the teeth for experimental study has not been indicated, the following experiments were designed to provide such information.

Experiment 1: Each of a group of 120 white rats weighing approximately 250 Gm. was injected intraperitoneally with 1 cc. of a solution containing 0.1 Gm. per cc. of radioactive potassium biphosphate (KH_2PO_4) with an activity of 200,000 counts per minute per cc. as measured in a cup 15 mm. in diameter 1.3 cm. from an Eck and Krebs counter tube. At the end of varying periods of time 15 rats were killed and the activity of the teeth determined in the following manner: From all rats two teeth were removed after boiling the heads in water for five minutes to aid removal. The teeth were washed with 0.5% potassium hydroxide and water, and dried; the teeth were then ground to a fine powder, and a quantity weighed into a standard counting cup 15 mm. in diameter. Two drops of 20% sulfuric acid were added and the residue dried in an oven at 140° after which it was ashed in a muffle furnace at 550° to 600°. The activity of the residue was then determined by a Geiger-Muller counter. All counts were corrected to a standard time. The results of the rate of phosphorus uptake are given in Table I.

TABLE I.—RATE OF PHOSPHORUS UPTAKE IN RATS' TEETH ON INTRAPERITONEAL INJECTION OF PHOSPHATE

Rat No.	Time Killed After Injection, Hr.	Average Activity per 100 Mg. of Tooth, C. p. m.[a]
1–15	1/2	52.6
16–30	1	55.9
31–45	2	72.1
46–60	5	88.1
61–75	10	101.0
76–90	20	287.5
91–105	53	98.2
106–120	66	108.3

[a] C. p. m. = counts per minute.

These results indicate that the phosphorus after a single intraperitoneal injection of phosphate is taken up in the teeth quite rapidly in the first twenty hours, after which the activity of the teeth diminishes.

Experiment 2: This experiment was designed to determine the activity of radioactive potassium biphosphate necessary to produce a desirable activity in the rats' teeth for experimental purposes in the study of mineral depletion. A group of 160 white rats, weighing about 250 Gm., was injected with 1 cc. of a solution containing 0.1 Gm. of radioactive potassium biphosphate of varying radioactivity. All rats were killed at the end of twenty hours and the activity of the teeth determined as in the preceding experiment. Table II gives the results obtained.

TABLE II.—THE ACTIVITY OF RATS' TEETH AFTER INTRAPERITONEAL INJECTION OF PHOSPHATE SOLUTION OF VARYING RADIOACTIVITY

Rat No.	Activity of Phosphate, C. p. m.[a] per Ml.	Average Activity per 100 Mg. of Tooth C. p. m.[a]
1–20	233,412	629
21–40	438,664	761
41–60	594,823	782
61–80	854,238	984
81–100	1,329,425	1156
101–120	1,998,511	1739
121–140	1,318,216	3265
141–160	6,274,742	4882

[a] C. p. m. = counts per minute.

These data show conclusively that the activity of rats' teeth after injection intraperitoneally of radioactive phosphate is a linear function of the activity of the phosphate solution injected.

SUMMARY

The purpose of this study was to determine the rate of uptake of phosphate in rats' teeth on intraperitoneal injection of phosphate and to determine the phosphate activity necessary to incorporate sufficient activity in the teeth for phosphorus depletion studies. The results indicate

that the most economical, rapid, and efficient method for obtaining rat teeth containing sufficient radioactive phosphorus to insure at least 35 counts per minute per mg. (as measured in a 15-mm. cup 1.5 cm. from an Eck and Krebs counter tube) is to inject 250-Gm. rats intraperitoneally with 1 cc. of a radioactive potassium biphosphate solution containing 0.1 mg. per cc. and having an activity of between five and seven million counts per minute per cc., and remove the teeth twenty hours after injection. Teeth containing an activity of at least 35 counts per minute per mg. have been shown to be satisfactory for studies involving the phosphorus depletion of teeth.

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Book Reviews

Clinical Toxicology. 2nd ed. By CLINTON H. THIENES and THOMAS J. HALEY. Lea and Febiger, Philadelphia, 1948. 373 pp. 13 x 20 cm. Price $4.75.

The modern approach to toxicology is an experimental one and this has led to many practical, life-saving benefits. Dr. Thienes' book reflects this approach and consequently it is essentially factual. Several points brought out are suggestive of further research needed. For example, in view of the biphasic character of morphine action on the central nervous system one wonders about the advisability of using caffeine to overcome respiratory depression.

This revision of *Clinical Toxicology* introduces a wealth of new material on such things as DDT, thiouracil, BAL, streptomycin, and dicumarol and includes uses of proteins, amino acids, vitamins, etc., in treatment of poisoning. The section on chemical identity of poisons has been enlarged also and new methods for preserving tissues after autopsy have been introduced.

If pharmacists find it impossible to have more than one book in this field, this one should be given highest consideration for the book of choice.

Hemostatic Agents with Particular Reference to Thrombin, Fibrinogen, and Absorbable Cellulose. By WALTER H. SEEGERS and ELWOOD A. SHARP. Charles C. Thomas, Springfield, Illinois, 1948. xii + 131 pp. 15 x 23 cm. Price $4.50.

The mechanism of the clotting of blood has never been completely understood, a fact which led to complex theories not always easy to comprehend. The authors of this book have given a very clear account of this physiological mechanism.

Although it was just ten years ago that Seegers, Warner, Brinkhous, and Smith first used thrombin successfully as a hemostatic agent in animals, it is frequently used in surgery today. Its use is discussed briefly in this stimulating book, as is also the use of fibrinogen. Modern application of such products as oxidized cellulose, fibrin foam, and gelatin sponge is covered also.

All of the many complex problems of research and development of hemostatic agents have not been solved. Such a carefully documented review (370 references) will be helpful to those interested in following the important developments in this area.

Life, Its Nature and Origin. By JEROME ALEXANDER. Reinhold Publishing Company, New York, 1948. viii + 291 pp. 14 x 23 cm. Price $5.00.

It was in the spring of 1899 when young Jerome Alexander submitted a thesis for his master's degree entitled "The Importance and Trend of Recent Work on the Chemistry of Life and the Products of Life." During all of the intervening years Dr. Alexander has been keenly interested in this subject although his major professional career has been in the colloid field. The author has integrated a vast amount of scientific literature into a picture of biocatalysis that probably accounts for as accurate an account of the physical origin of life as is possible with today's knowledge.

Those interested in a philosophic interpretation of science will find Dr. Alexander's book uplifting and stimulating.

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